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(71) Applicant *(for all designated States except US)*: TEXAS A & M UNIVERSITY [US/US]; 310 Wisebaker, College Station, TX 77843 (US).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: DE GUZMAN MIROV, Greta, J. [PH/PH]; 2810 E Zobel Street, Makati City 1200 (PH). KELLY, Jeffery, W. [US/US]; 213 Chimney Hill, College Station, TX 77840 (US). LAI, Zhihong [CN/US]; 3216 Hunter's Glen Drive, Plainsboro, NJ 08536 (US). LASHUEL, Hilal, A. [US/US]; Apartment 6, 198 Court Street, Brooklyn, NY 11201 (US). PETERSON, Scott, A. [US/US]; 3723 Cobblestone Place, Topeka, KS 66610 (US).

(74) Agent: FREEMAN, John, W.; Fish & Richardson P.C., 225 Franklin Street, Boston, MA 02110–2804 (US).

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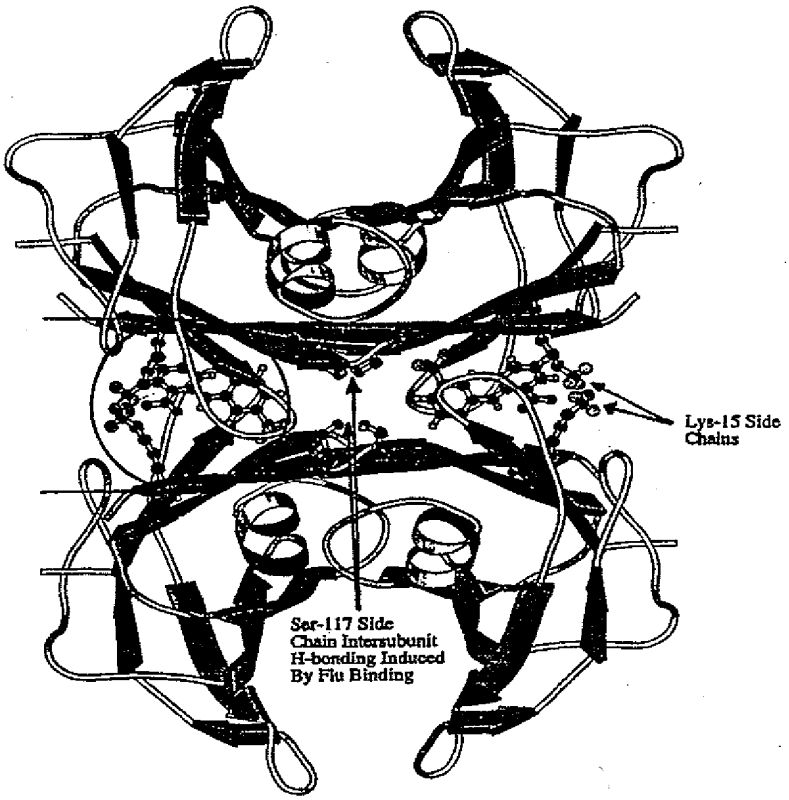
(57) Abstract

A method for treating a human amyloid disease which includes administering a pharmaceutically effective amount of a composition including an amyloidogenic protein–stabilizing aryl compound.



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ANTI-AMYLOIDOGENIC AGENTS

Statement as to Federally Sponsored Research

This invention was made with support from the National Institutes of Health (Grant R29 DK46335-01). Accordingly, the U.S. government may have certain rights in this invention.

Background of the Invention

The invention features anti-amyloidogenic compounds, and methods of using them.

Amyloidosis is characterized by the extracellular deposition in tissue of fibrillar proteins with a β-pleated sheet conformation. Fibrillar proteins are relatively insoluble and resistant to proteolytic digestion.

Transthyretin (TTR), also known as thyroxine binding prealbumin, is found in human plasma as a tetramer of identical 127 residue subunits. Each TTR subunit contains 1 cysteine residue which does not participate in disulfide formation. Each TTR subunit adopts a predominantly β-sheet structure. One four-stranded β-sheet interacts face-to-face with another four-stranded β-sheet, forming a β-sheet sandwich monomer that is the hydrophobic core of the protein. The β-sheet sandwich monomers dimerize through an intermolecular antiparallel β-sheet interaction to form an eight-stranded β-sandwich dimer. Two such dimers form the TTR tetramer.

Normally, tetrameric TTR is the mature fold of the protein. However, in amyloid disease states, tetrameric TTR can be converted into an insoluble fibrillar quaternary structure (an amyloid fibril). The conversion can occur by partial denaturation of the tetramer which

exposes interior residues of each of the four monomers which interact with corresponding residues on other partially-unfolded monomers. Amyloid fibrils are formed from amyloidogenic intermediates under mildly denaturing conditions. An amyloid fibril is an insoluble deposit of an otherwise soluble protein or protein fragments which self-assemble into fibrils about 100 Å in diameter and of variable length. There are at least 16 known human amyloidogenic proteins which have little sequence homology, and yet all are able to form a similar amyloid fibril. Amyloid fibrils may be neurotoxic or may interfere with normal organ function, thereby contributing to a variety of clinical syndromes.

Current therapies for systemic amyloid diseases attempt to remove the source of the precipitating β-protein with drugs that inhibit protein synthesis. In familial amyloid polyneuropathy, for example, a liver transplant is intended to replace the mutant form of TTR produced by the liver, as the mutant form is more readily converted into amyloid in vivo. Current therapies for Alzheimer's disease includes acetylcholinesterase inhibitors which are intended to inhibit the degradation of acetylcholine.

Other diseases associated with amyloid fibril formation are Alzheimer's disease, familial amyooid polyneuropathy (FAP), and senile systemic amyoidosis (SSA).

Summary of the Invention

The invention features a method for treating a human amyloid disease, which method includes administering a pharmaceutically effective amount of a composition including an amyloidogenic protein-stabilizing compound having the formula (1a), (1a'), (1b), (1c), (1d), or the formula of a nonsteroidal anti-

inflammatory agent described in greater detail in the claims.

The disease can be selected from primary systemic amyloidosis, senile systemic amyloidosis, familial amyloid polyneuropathy I, hereditary cerebral amyloid angiopathy, hemodialysis-related amyloidosis, familial amyloid polyneuropathy III, Finnish hereditary systemic amyloidosis, Type II diabetes, medullary carcinoma of the thyroid, spongiform encephalopathy, atrial amyloidosis, hereditary non-neuropathic systemic amyloidosis, injection-localized amyloidosis, and hereditary renal amyloidosis. Preferably the disease is senile systemic amyloidosis or familial amyloid polyneuropathy I; or Alzheimer's disease; or spongiform encephalopathy. In one embodiment, the amyloidogenic protein is transthyretin or a variant thereof.

According to the disclosed method, the compound has the formula (1a), (1a'), (1b), (1c), (1d), or is selected from the individually disclosed compounds. For example, the compound is fenoprofen or flufenamic acid. The invention also features those disclosed compounds which have novel structures.

In one embodiment, an amyloidogenic protein-stabilizing compound binds to transthyretin *in vitro* with a higher affinity than it binds to a human serum binding protein, such as thyroxine binding globulin or serum albumin. The compound may also bind to transthyretin *in vitro* with a higher affinity than it binds to thyroid hormone receptor.

The invention also includes an amyloidogenic protein-drug conjugate. This conjugate includes an amyloidogenic protein reversibly bound to a compound of formulae (1a), (1a'), (1b), (1c), or (1d). Examples of compounds include compounds selected from a

biphenylether, a biphenylthioether or a biphenylamine, such as fenoprofen or flufenamic acid.

The invention also features a method for inhibiting amyloid fibril assembly which includes forming an amyloidogenic protein/drug conjugate, and a method for stabilizing an amyloidogenic protein which includes exposing a target protein to a disclosed compound (e.g., administering a pharmaceutically effective amount of a composition including an amyloidogenic protein-stabilizing compound to a patient, or exposing the target protein *in vitro*). This method for stabilizing an amyloidogenic protein can further include the step of forming an amyloidogenic protein/drug conjugate. Also disclosed are methods for screening for substances which inhibit amyloid fibril assembly by stabilizing an amyloidogenic protein.

Brief Description of the Figures

Fig. 1 is a ball-and-stick model of the flufenamic binding site of TTR. Only residues that line the binding cavity are displayed. Flufenamic acid is shown in one of the two binding modes. Because of the two-fold symmetry along the binding channel two molecules fit the final $2|F_o|-|F_{c\ calc}|$. The CF_3 substituent occupies the innermost T4 hologen binding pocket, interacting with Ser-117, Thr-119, Leu-110 and Ala-108 providing hydrophilic and hydrophobic fluorine contacts. The aromatic rings interact with the hydrophobic patch between the residues Leu-17 and Ala-108. Finally the carboxylate group on the outer phenyl ring is placed at the entrance of hte funnel-shaped binding pocket forming electrostatic interactions with two Lys-15 residues. The image has been generated using the program O (18).

Figure 2 shows that upon binding of flufenamic acid, the side chans of Ser-117, Thr-119, and Lys-15 undergo conformational changes as indicated. The Ser-117

residues particiate in intersubunit hydrogen bonding while the Thr-119 side rotates to form a hydrogen bond to an ordered water molecule which is hydrogen bonded to carbonyl oxygen of Asp-18' on an adjacent subunit.

Detailed Description of the Invention

The invention features a method for treating a protein deposition disease (e.g., an amyloid disease or a light chain deposition disease) which method includes administering a composition which includes an protein assembly inhibitor compound (e.g., an amyloidogenic protein-stabilizing compound), thereby inhibiting protein deposition (e.g., amyloid fibril formation).

Human amyloid diseases, also known as cross-β amyloid fibril-mediated diseases, are defined by the presence of extracellular amyloid deposits which appear to cause disease. Amyloid deposition contributes to neurotoxicity and/or crowds out normal tissue in a given organ, resulting in organ dysfunction. These amyloid diseases can therefore be systemic or neural, and include Alzheimer's disease, primary systemic amyloidosis, senile systemic amyloidosis, familial amyloid polyneuropathy I, hereditary cerebral amyloid angiopathy, hemodialysis-related amyloidosis, familial amyloid polyneuropathy III, Finnish hereditary systemic amyloidosis, Type II diabetes, medullary carcinoma of the thyroid, spongiform encephalopathy, atrial amyloidosis, hereditary non-neuropathic systemic amyloidosis, injection-localized amyloidosis, and hereditary renal amyloidosis. Other diseases include light chain deposition disease (non-amyloid deposits), and prion disease, such as mad cow disease.

Some corresponding amyloidogenic precursor proteins (and fibril components) are, respectively: β protein (β protein 1-40, 1-41, 1-42, and 1-43), immunoglobin light chain (intact light chain or fragments

thereof) (several single site variants), serum amyloid A (amyloid A (76 residue fragment), transthyretin (transthyretin or fragments thereof), transthyretin (over 45 transthyretin variants), cystatin C (cystatin C minus 10 residues), β_2-microglobulin (same), apolipoprotein A-1 (fragments thereof), gelsolin (71 amino acid fragment thereof), Islet amyloid polypeptide or IAPP (fragment of IAPP), calcitonin (fragments thereof), prion (prion or fragments thereof), atrial natriuretic factor or ANF (same), lysozyme (lysozyme or fragments thereof), insulin (same), and fibrinogen (fragments thereof). In some embodiments, the amyloidogenic protein is selected from any of the above, excluding lysozyme, insulin, or both.

An amyloidogenic protein is a precursor protein, or fragment thereof, which can be converted into an insoluble amyloid fibril *in vivo*. Amyloidogenic proteins include a non-fibril conformation, the normally-folded amyloid protein, or a tertiary or quaternary structure that precedes the amyloid such as an oligomer or partially folded protein in the case of a proteolytic fragment of an amyloidogenic protein. Amyloidogenic proteins are generally soluble and include nonnative quaternary structures that precede amyloid fibril assembly, such as a 16-mer, a 44-mer, or a 64-mer. Amyloidogenic proteins also include fibril components (as listed in parentheses above).

An amyloidogenic protein-stabilizing compound can act by binding reversibly, and preferably selectively, to one or more amyloidogenic proteins. Binding to a protein can stabilize an existing protein conformation, or render the protein resistant to the abnormal degradation or partial denaturation which results in amyloid formation or deposition. The binding of the amyloid stabilizing compound to any of the above reduces, in part or whole,

the overall formation of amyloid. Examples of amyloid stabilizing compounds are formulae (1a), (1b), (1c), and (1d). An amyloidogenic protein-stabilizing compound can also act by irreversible binding (covalent linkage) to the amyloidogenic protein.

An amyloidogenic protein-stabilizing compound preferably binds to an amyloidogenic protein, e.g., transthyretin, with a higher affinity that it binds to a non-amyloidogenic protein. In this context, a non-amyloidogenic protein is a protein or receptor, such as a hormone receptor, a human serum binding protein, or an amyloid fibril. A higher affinity refers to a K_d relative to amyloidogenic protein at least 5 times lower (e.g., about one or more magnitudes lower) than the K_d of the non-amyloidogenic protein.

Regarding the higher affinity, it should be noted that TTR liver transplant studies have indicated that TTR amyloid can be cleared *in vivo*. Thus, the combination of the rate of clearance of amyloidogenic proteins (e.g., the quaternary structures such as a 16-mer) and the rate of fibril formation are considered together. Depending on the clinically effective threshold ratio of an amyloidogenic protein/drug conjugate to free amyloidogenic protein, it may be sufficiently effective for an amyloidogenic protein-stabilizing compound to slow down amyloid fibril assembly, rather than substantially stop such assembly. For example, if 20%, 30%, or 40% of the free amyloidogenic protein (e.g., TTR) is in conjugate form, a stabilizing compound having a K_d that is not even one magnitude lower can be effective. An amyloidogenic protein-drug conjugate includes a drug, e.g., an amyloidogenic protein-stabilizing compound reversibly bound to an amyloidogenic protein. Preferably, the reversibly binding is with high affinity (low K_d), and with a low off-rate relative to on-rate so

the drug occupies the amyloidogenic protein for an effective period of time.

Transthyretin includes the wild type protein and any of the more than fifty variants having one or more mutations that are associated with the onset of either senile systemic amyloidosis or familial amyloid polyneuropathy, such as Val-30-Met mutation. Although most single site mutations do not significantly affect the tertiary or quaternary structure of tetrameric TTR, they may nevertheless destabilize the protein. A variant can be used in an *in vitro* assay wherein a disclosed compound binds to TTR with a higher affinity than a serum protein.

Human serum binding proteins include thyroxine binding globulin (TBG), serum albumin, globulins, transferrin, ceruplasmin, glycoproteins, α-lipoproteins, and β-lipoproteins. Thyroid hormone receptors generally regulate growth, development, and metabolic rates in different tissues including cardiovascular, skeletal, gastrointestinal, and neuromuscular systems. Thyroid metabolites of thyroxine such as T3 and agonists or antagonists thereof bind to thyroid hormone receptors. Excessive exposure to thyroid hormone agonists may result in, for example, bradycardia, menstrual abnormalities, weight gain, or anxiety. To avoid undesirable secondary effects, a amyloidogenic protein-stabilizing compound preferably has a relatively low affinity for a thyroid hormone receptors, e.g., binds with a K_d that is at least one order of magnitude, and preferably 1.5, 2, 2.5, or more orders of magnitude higher than the K_d for thyroid metabolites such as T3.

Embodiments

The compounds disclosed herein which inhibit intermolecular aggregation can be used as therapeutics

for any disease or condition that is mediated by an amyloid protein which requires assembly, such as self-assembly of dimeric or oligomeric forms for activity (e.g., neurotoxicity or organ dysfunction). One aspect of the invention therefore features a method of inhibiting amyloid protein assembly, including exposing an aqueous solution of amyloid protein at physiological temperature and pH to an amyloidogenic protein-stabilizing compound. One embodiment further includes measuring the extent of amyloid protein assembly. Turning to an example using a protein precursor such as the oligomeric protein transthyretin (TTR), in one embodiment a preferred compound inhibits TTR fibril formation (Example 7). Sedimentation velocity experiments demonstrated that TTR undergoes conformational change-mediated dissociation to form a monomeric amyloidogenic intermediate which self-assembles into amyloid in the absence of the inhibitor compound (Example 6), but not in its presence (binds to transthyretin, but does not bind in a pharmacologically significant (or adverse) way to thyroxine binding globulin (TBG) (Example 9), a thyroid hormone receptor (i.e., is not a potent thyroxine agonist or antagonist) (Example 10), or other plasma proteins.

Additional *in vitro* assays and animal disease models are known to those in the art, such as those described in Munro, et al., *J. Clin. Endocrinol. and Metab.* 68:1141-1147 (1989) (TTR binding) and Merlini, et al., *Proc. Natl. Acad. Sci.*, 92:2959-2963 (1995) (experimental amyloid murine model, 4'-iodo-4'-deoxydoxorubicin binds to immunoglobulin light chains). Transgenic mice useful for evaluating therapeutics in transthyretin amyloid disease have also been developed by Yamamura et al. *Mol. Biol. Med.* 6:333-343 (1989), and

Buxbaum et al. *Amyloid: Int. J. Exp. Clin. Invest.* 3:187-208 (1996).

Another aspect of the invention features a method of inhibiting intermolecular protein assembly, including exposing a target protein to a disclosed compound. The protein can be in solution, in fibrillar form, or in crystalline form. In one embodiment, the target protein requires monomeric form for function, and is thereby stabilized. In another embodiment, the target protein requires dimeric or tetrameric form for function, and its function is thereby inhibited. In some embodiments, inhibition of protein assembly is useful for storage or analysis of a protein. Similarly, the invention includes a method of inhibiting amyloid protein assembly, comprising exposing an aqueous solution of amyloid protein at physiological temperature to a disclosed peptidomimetic. In some embodiments, the aqueous solution has a pH between 5.0 and 7.5; or the method further includes, after the exposing step, the step of measuring the extent of amyloid protein assembly.

The invention also features a method of down-regulating a target amyloidogenic protein, including administering to a patient an effective amount of a pharmaceutical composition containing containing a disclosed compound. One embodiment of this method reduces the incidence of interaction between the protein and its receptor by interfering with protein-receptor recognition.

Another aspect of the invention is a method of imaging an amyloidogenic protein *in vivo*, including administering a detectable label (e.g., radiolabel or fluorescent label) linked to a disclosed compound, and detecting said label. One embodiment of this method distinguishes between active (e.g., functional or pathological) conformations or assemblies and inactive or

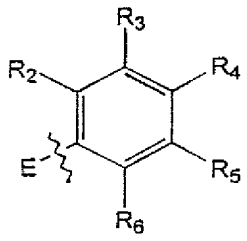
precursor conformations. The link can be a covalent bond or an antibody-antigen interaction.

The invention also includes a method for treating a human light chain deposition disease, comprising administering a pharmaceutically effective amount of a composition including a deposition-inhibiting compound having a formula selected from the formulae (1a), (1a'), (1b), (1c), (1d), and a non-steroidal anti-inflammatory drug.

The invention also features methods of making medicaments for treating human amyloid diseases or conditions characterized, e.g, those by undesired amyloid protein assembly by formulating the designated compounds with a pharmacologically acceptable carrier.

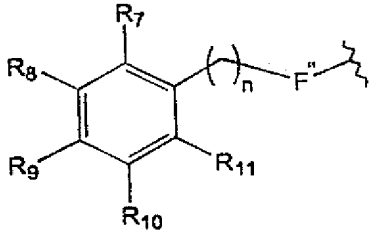
Compounds

The compounds used in the disclosed methods include those of formula (1a):

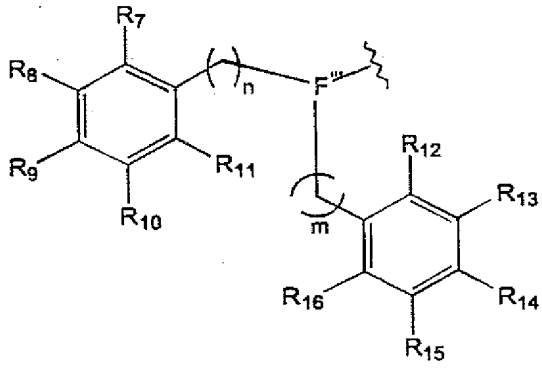


(1a)

wherein E is -F'-R_{17}, formula (i), or formula (ii)



(i)

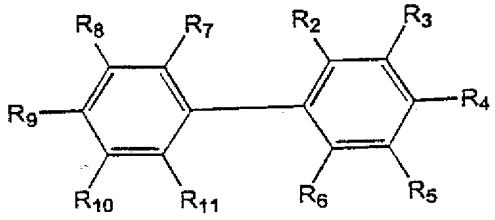


(ii)

in which

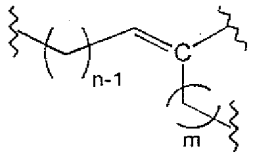
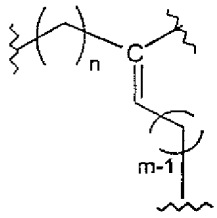
F' is NR_a, O, or S;

F" is NR_a, O, S, CH_2, $Si(R_b)(R_c)$, $-PR_b$, or $[-F"-(CH_2)_n-]$ is deleted such that a amyloidogenic protein-stabilizing compound (1a') results;

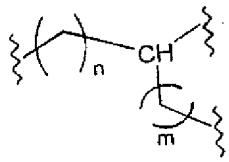


(1a')

F''' is N, sp^2 hybridized C,

i.e.,  or  ,

or sp^3 hybridized C-H,

i.e.,  ;

R_a is H or C_{1-6} alkyl;

each of R_b and R_c is independently C_{1-6} alkyl;

provided that at least two of R_1 - R_{17} are each independently selected from halo, C_{0-8} hydroxyalkyl, or -A-B,

wherein A is branched or straight C_{0-6} alkylene, C_{1-6} acyl, or C_{1-6} aminoalkylene;

B is R_d, $-COOR_d$, $-OR_d$, $-SR_d$, $-NHR_d$, or $-N(C_{1-3}$ alkyl$)(R_d)$;

R_d being H, straight or branched C_{1-8} alkyl, C_{2-8} heterocyclic radical, C_{2-8} heteroaryl, or (C_{0-4} alkyl)phenyl;

and the remaining R_1 - R_{17} are independently selected from H, C_{1-6} alkyl, C_{1-6} haloalkyl, benzyl, C_{1-8} alkene, C_{3-8} cycloalkyl, $N^+R_fR_gR_h$,

each of R_f, R_g, and R_h being independently selected from H and C_{1-10} alkyl, (C_{0-4} alkyl)$-SO_3H$, $-(C_{0-4}$ alkyl$)-PO_3H_2$, $-(C=O)(C_{1-5}$ alkyl), and $-R_j-M-R_k$, where R_j is straight or branched C_{1-10} alkylene; R_k is H, or straight or branched C_{1-10} alkyl; and M is carbonyl or a heteroatom; or R_j-M-R_k taken together are C_{4-10} cycloalkyl, C_{2-8} heterocyclic radical, or C_{2-8} heteroaryl;

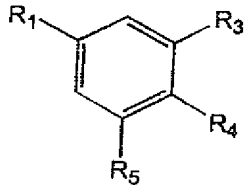
each of n and m is independently an integer between 0 and 2, inclusive (e.g., between 0 and 1). In another aspect, a proximate pair of substituents, such as R_2 and R_7 or R_6 and R_{11}, can be taken together to form a covalent bond.

Representative examples of formula (1a) are shown in Scheme D.

In certain embodiments of formula (1a), there are at least two, at least three, or at least four non-hydrogen ring substituents on each ring or on all rings combined.

In some embodiments, halo is F, Br, or preferably I, e.g., in a mono-iodo or diiodo compound; or there is an acidic group meta or para to the heteroatom (e.g., ether oxygen).

Another compound used in the disclosed methods is a compound of formula (1d)



(1d)

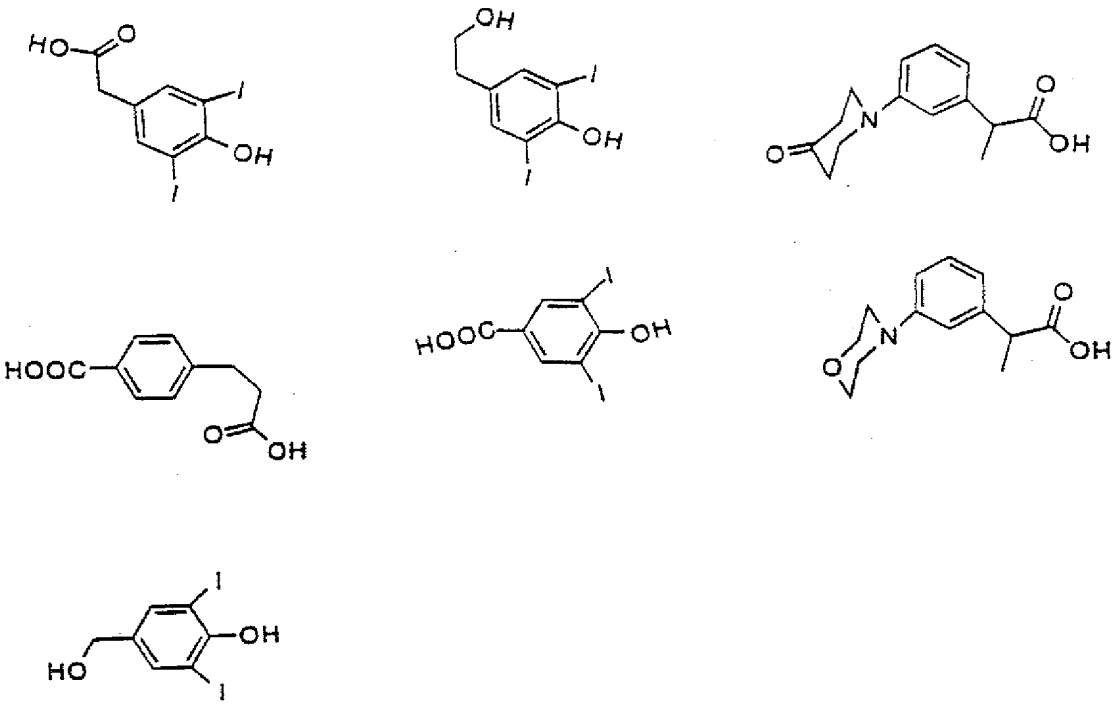
At least one of R_1 and R_4 is selected from F, Cl, Br, I, hydroxy, C_{1-6} haloalkyl (e.g., trifluoromethyl), C_{1-8} alkyl,

-A-B where A is branched or straight C_{0-6} alkylene, C_{1-6} acyl, or C_{1-6} aminoalkylene, and B is R_a, $-COOR_a$, $-OR_a$, $-SR_a$, NHR_a, or $N(C_{1-3}$ alkyl$)(R_a)$, R_a being H, straight or branched C_{1-8} alkyl, C_{2-8} heterocyclic radical, C_{2-8} heteroaryl, or (C_{0-4} alkyl)phenyl. Each of R_3, R_5, R_6, and any remaining R_1 or R_4 is independently selected from H, halo (e.g., I or Br), hydroxy, C_{2-8} heterocyclic radical, C_{2-8} heteroaryl, and cyano. Representative compounds of formula (1d) are shown in Scheme A.

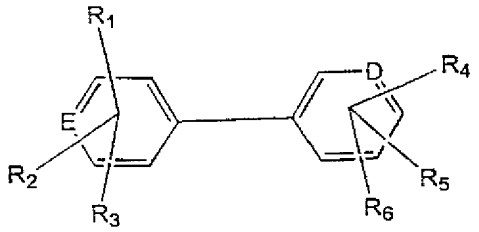
In some embodiments of formula (1d), one of R_1 and R_4 is selected from F, Cl, Br, I, hydroxy, C_{1-6} haloalkyl (e.g., trifluoromethyl), C_{1-8} alkyl, -A-B where A is C_{0-3}

alkylene, C_{1-4} acyl, or C_{1-4} aminoalkylene, and B is R_a, -$COOR_a$, or -OR_a, R_a being H, C_{1-5} alkyl, C_{2-6} heterocyclic radical, or C_{2-5} alkoxyalkyl. R_6 is H and each of R_3, R_5, and any remaining R_1 or R_4 is independently selected from H, I, Br, hydroxy, C_{2-6} heterocyclic radical, and cyano.

Scheme A



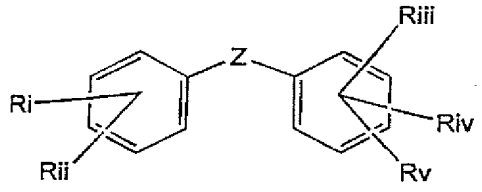
Yet another compound used in the disclosed methods has the formula (1b):



(1b)

Each of D and E is independently selected from C, N, O, and S. No more than two of R_1 - R_6 are independently selected from -A-B where A is branched or straight C_{0-6} alkylene, C_{1-6} acyl, or C_{1-6} aminoalkylene, and B is R_a, -$COOR_a$, -OR_a, -SR_a, NHR_a, or $N(C_{1-3}$ alkyl$)(R_a)$, R_a being H, straight or branched C_{1-8} alkyl, C_{2-8} heterocyclic radical, C_{2-8} heteroaryl, or (C_{0-4} alkyl)phenyl. The remaining R_1 - R_6 are independently selected from H, halo, hydroxyl, and cyano. In general, non-hydrogen R_1 - R_6 are preferably in the *meta* or *para* positions relative to the other ring. In some embodiments, electron-withdrawing groups are in one or more of the four available *meta* positions (two on each ring). Representative compounds within formula (1b) are shown in Scheme B.

Other compounds have the formula (1c):

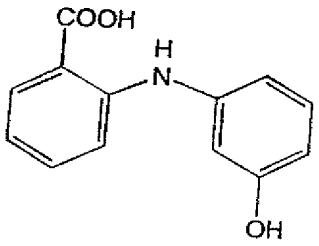


(1c)

Z is O or N(G), where G is selected from H, C_{1-8} alkyl, C_{5-10} cycloalkyl, C_{6-10} aryl, C_{2-8} heterocyclic radical, G being optionally substituted with one or more halo, hydroxy,

carboxyl, C_{1-2} haloalkyl, or cyano groups. Each of R_1 - R_5 is independently selected from hydrogen, halo, hydroxy, C_{1-5} alkyl, C_{1-5} haloalkyl, cyano, and -A-B as described in formula (1a). In some embodiments, non-hydrogen ring substituents (e.g., electron-withdrawing substituents) are preferably *meta* or *para*; non-hydrogen ring substituents can also be in an *ortho/para*, *ortho/ortho*, or *ortho/para/ortho* arrangement. Representative compounds of formula (1c) are shown in Scheme C.

The invention includes the disclosed method for treating human amyloid diseases using compositions which include known non-steroidal anti-inflammatory drugs (NSAIDs) such as 2,4,6-triiodophenol, mefenamic acid, diflunisal, meclofenamic acid, milrinone, ethacrynic acid, diclofenac, indomethacin, sulindac, fenclofenac, EMD 21388 (Merck), and preferably, fenoprofen and flufenamic acid (Schemes D and E). Flufenamic acid has the following formula:



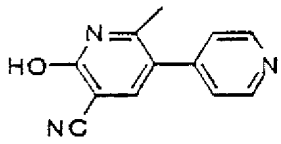
The invention also features metabolic products of disclosed compounds, and prodrugs which, when metabolized, result in a disclosed compound or one or more metabolic products of a disclosed compound. For example, esters, amines, ammonium salts, and Tris salts can be easily formed from the disclosed compounds.

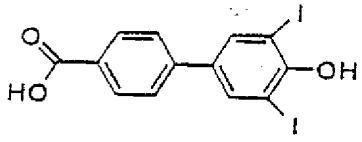
Alkyl groups include straight chain alkyls such as methyl, ethyl, propyl, butyl, and pentyl; branched alkyls

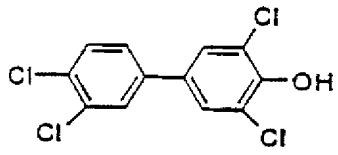
such as isopropyl, isobutyl, t-butyl, sec-pentyl, and 2-methyl-4-ethylheptyl; and cycloalkyls, such as cyclopentyl, cyclohexyl, and 2,4-dimethylcyclohexyl. C_{1-10} alkyls, such as C_{1-6} alkyls or C_{1-3} alkyls.

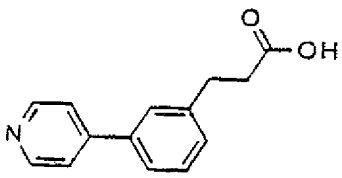
Alkenyl groups are C_{2-10}, e.g., C_{2-8} or C_{2-6}. Like alkyl groups, they include straight chain, branched, and

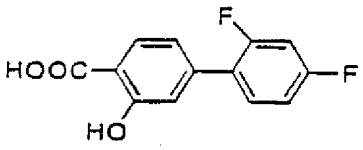
Scheme B

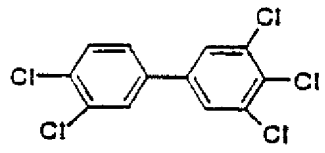


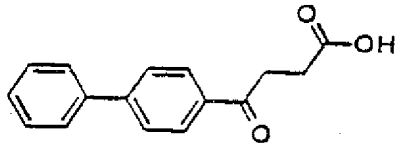




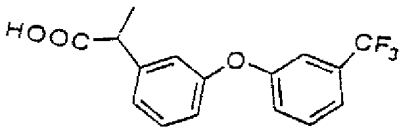


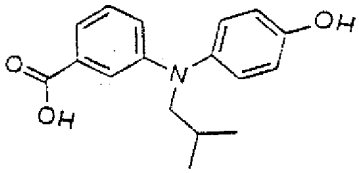


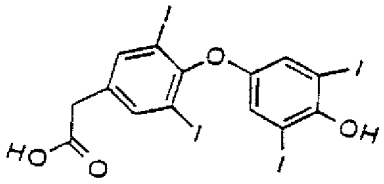


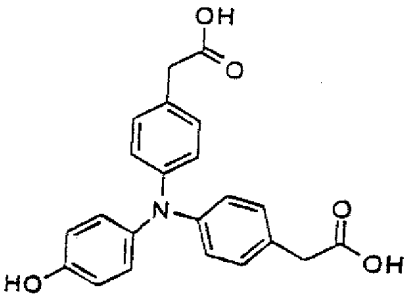


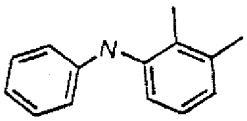
Scheme C

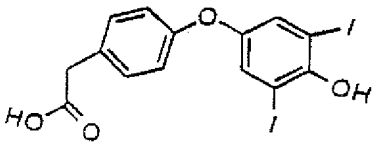

HOOC
O
CF3

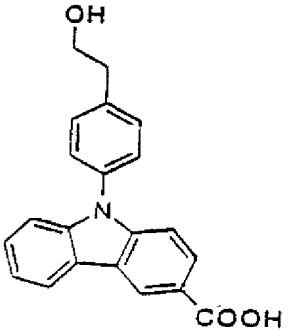

O
OH
N
OH

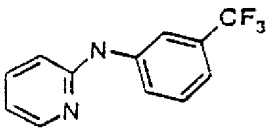

I
O
I
I
HO
O
I
OH


OH
O
N
O
HO
OH

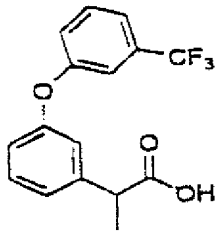

N

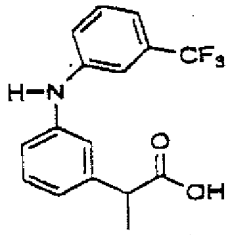

O
I
OH
HO
O
I

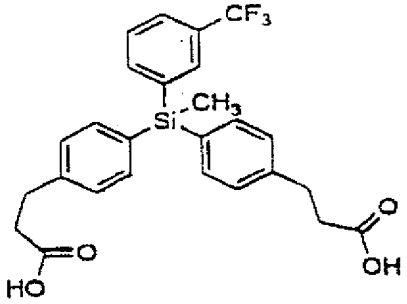

OH
N
COOH

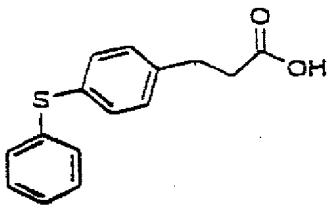

CF3
N
N

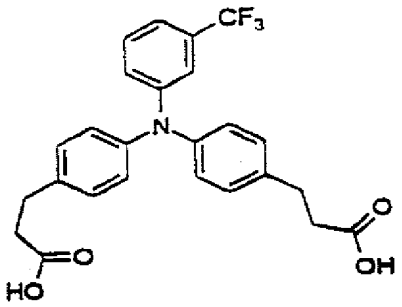
Scheme D

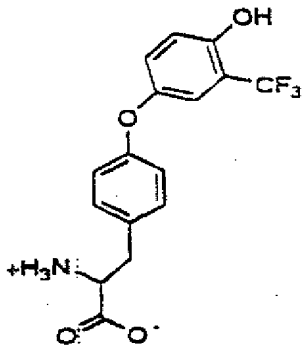

CF3
O
O
OH

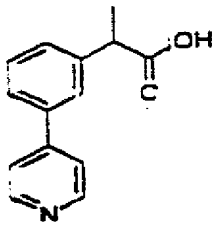

H-N
CF3
O
OH

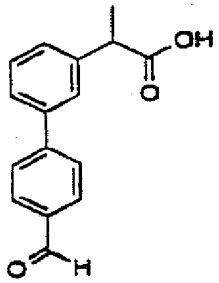

CF3
Si
CH3
O
HO
O
OH

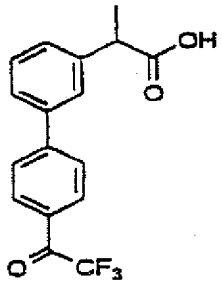

O
OH
S

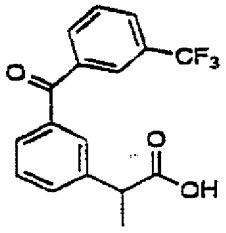

CF3
N
O
HO
O
OH

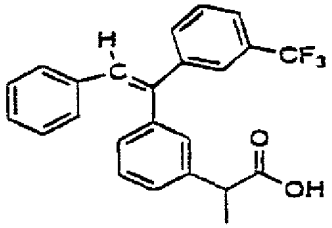

OH
CF3
O
+H3N
O
O


OH
O
N

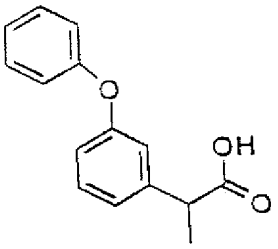

OH
O
O
H


OH
O
O
CF3

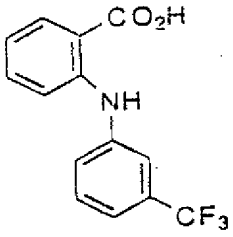

CF3
O
O
OH


H
C
CF3
O
OH

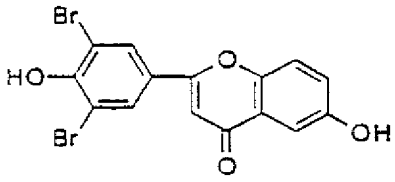
Scheme E



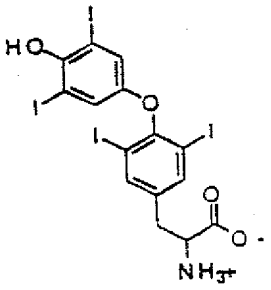
fenoprofen



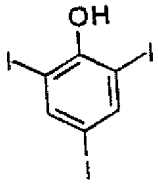
flufenamic acid



EMD 21388



thyroxine



triiodophenol

cyclic moieties. A given double bond may be cis, trans, entgegen, or zusammen. When two or more double bonds are present, they may be conjugated or unconjugated. Alkenyl includes any alkyl group with at least two adjacent hydrogen atoms removed, such as 1-methyl-but-2-enyl, 1,3-butadienyl, isopropenyl, octa-3,6-dienyl, allyl, vinyl, isoprenyl, and prenyl. Alkynyl groups are C_{2-10}, C_{2-8} or C_{2-6}. An alkynyl group may also include one or more double bonds and a triple bond.

Hydroxyalkyl (or hydroxyalkenyl or hydroxyalkynyl) includes any alkyl (or alkenyl or alkynyl) group wherein at least one hydrogen is replaced with a hydroxyl group. Where more than one hydrogen is replaced (e.g., wherein a hydroxy-alkyl is a diol or triol), the hydroxyl groups may be on the same carbon atom (*gem*-diol) or on different carbon atoms (e.g., 1,2-diol or 1,3-diol).

Haloalkyl (or haloalkenyl or haloalkynyl) includes any alkyl (or alkenyl or alkynyl) group wherein at least one hydrogen is replaced with a halogen (fluorine, chlorine, bromine, or iodine). Where more than one hydrogen is replaced (e.g., wherein haloalkyl is a dihaloalkyl or hexahaloalkyl), the halogens are selected independently. For example, halomethyl includes perchloromethyl ($-CF_3$), bromomethyl ($-CH_2Br$), and fluorochloromethyl ($-CHFCl$). Where more than one hydrogen is replaced, the halogens may be on the same carbon atom (e.g., perfluoroethyl) or on different carbon atoms (e.g., 2-iodo-3-bromobutyl). Amino-substituted and nitro-substituted alkyls (or alkenyls or alkynyls) are analogous to the above.

An aryl group is a C_{6-40} aromatic ring, wherein the ring is made of carbon atoms (e.g., C_{6-20}, C_{6-12}, or C_{6-10} aryl groups). Examples include phenyl, halophenyl, benzyl, naphthyl, binaphthyl, mesityl, tolyl, xylyl, azulyl, indyl, pentalyl, phenanthrenyl, and biphenylyl.

For example, C_8 aryl includes alkylaryls, alkenylaryls, alkynylaryls, arylalkenyls, arylalkynyls, and arylalkyls such as 4-methylbenzyl, 3-ethylphenyl, 4-vinylphenyl (4-ethenylphenyl), and 2,3-dimethylphenyl. (C_6 aryl)(C_4 alkanoyloxy) includes Ph-$(CH_2)_4$(C=O)O- and the (R) and (S) stereoisomers of Ph-$CH_2CH(CH_3)CH_2$(C=O)O-. (C_9 aryl)(C_4 alkanoyloxy) includes (p-ethyl)-Ph$(CH_2)_4$(C=O)O- and the stereoisomers of (2,4-dimethyl)phenyl-$CH_2CH(CH_3)CH_2$(C=O)O-. A heterocyclic radical contains at least one ring structure which contains carbon atoms and at least one heteroatom such as N, O, or S. A heteroaryl is an aromatic heterocyclic radical. Examples of heterocyclic radicals and heteroaryl groups include: thiazolyl, 2-thienyl, 3-thienyl, 3-furyl, furazanyl, 2H-pyran-3-yl, 1-isobenzofuranyl, 2H-chromen-3-yl, 2H-pyrrolyl, *N*-pyrrolyl, imidazolyl, pyrazolyl, isothiazolyl, isoxazolyl, pyridyl, pyrazinyl, pyrimidinyl, pyridazinyl, indolizinyl, isoindolyl, indolyl, indazolyl, purinyl, phthalazinyl, cinnolinyl, and pteridinyl. For example, a C_{3-12} heteroaryl group may be a C_{3-6}, or a C_{4-9} group. A heterocyclic or heteroaryl radical may be attached to another moiety via a carbon atom or a heteroatom of the heterocyclic or heteroaryl radical.

Aminoalkyls or aminoalkylenes are alkyl or alkylene groups that include substitution with one, two, or more amino (-NH_2) groups. The base alkyl or alkylene can be straight chain or branched, as in thyroxine; an amino group may be charged (e.g., an ammonium salt). Aminoalkyls and aminoalkylenes also include secondary amines substituted with -NHR.

Synthesis

The classical Ullman ether synthesis procedure for making diaryl ethers involves the reaction of an aryloxide with an aryl halide (I≈Br > Cl >> F) (Weingarten, H., Ullman Condensation; *J. Org. Chem.* **29**:977-978 (1964); and Moroz et al., The Ullman Ether Condensation; *Russian Chemical Reviews* **43**:679-689 (1974)) in the presence of a copper salt, typically CuBr, CuBr-SMe_2, or CuCl, in a solvent such as diglyme or xylene. (Lindley, J., Copper Assisted Nucleophilic Substitution of Aryl Halogen; *Tetrahedron* **40**, 1433-1456 (1984)). This method provides simple aryl ethers and will work for preparing those inhibitors that do not contain one or more sensitive functionalities, i.e., a functionality that will decompose in basic solvents in the presence of copper salts at high temperatures (e.g., 160°C) for extended reaction times (e.g., 15 hours). (Moroz, A.A. et al. (1974)).

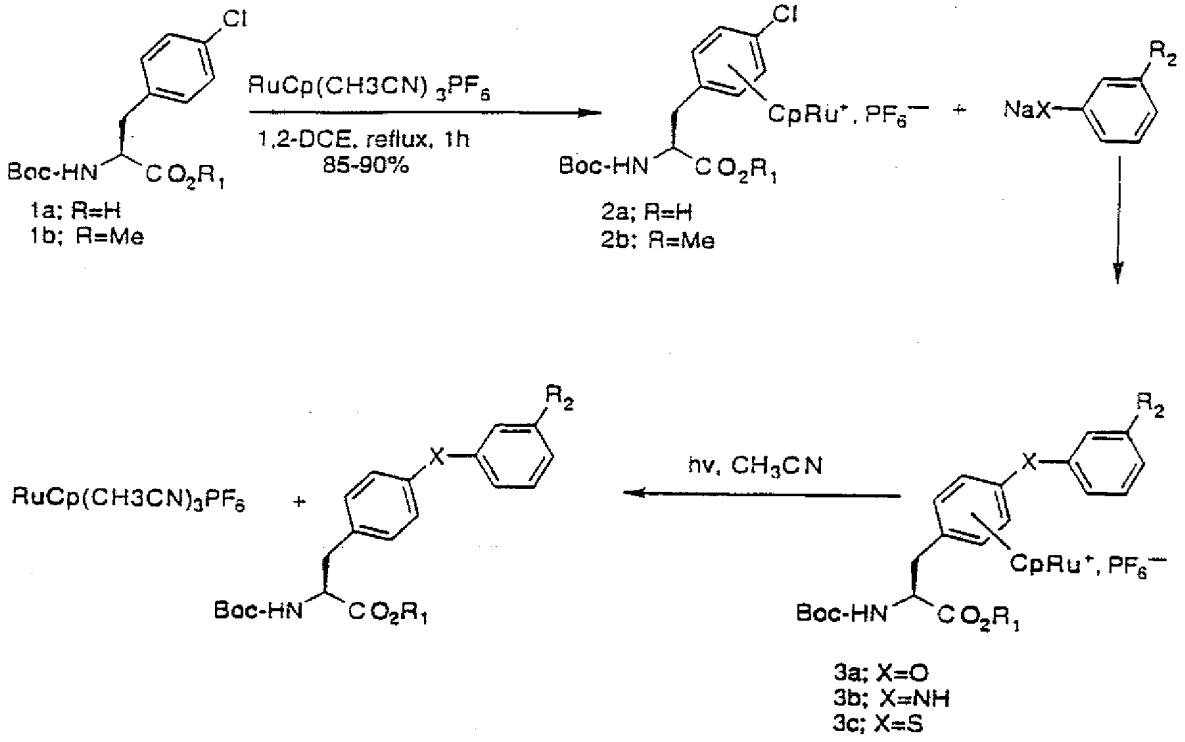
Diarylsulfides can be prepared by reacting the analogous arylsulfide (ArS-) with an aryl halide as described above using polar aprotic solvents. Lindley, (1984). Unactivated aryl halides also give good yields of aryl sulfides on treatment of ArS- in the presence of a catalytic amount of $(Ph_3P)_4Pd$. Diaryl sulfides can also be prepared in high yields by treatment of aryl halides with ArS- in liquid ammonia under irradiation. The Ullman approach also provides aryl amines from a reaction between an aryl halide and a primary amine, a secondary amine, or an aniline. Lindley, (1984). Additional methods for making ethers, thioethers and amines include phenolate displacement of I from aryliodonium salts, and the oxidative phenolic coupling methods of Yamamura and Evans, although these methods are less general.

Newer synthetic methods for the selective production of aryl ethers, sulfides and amines involve

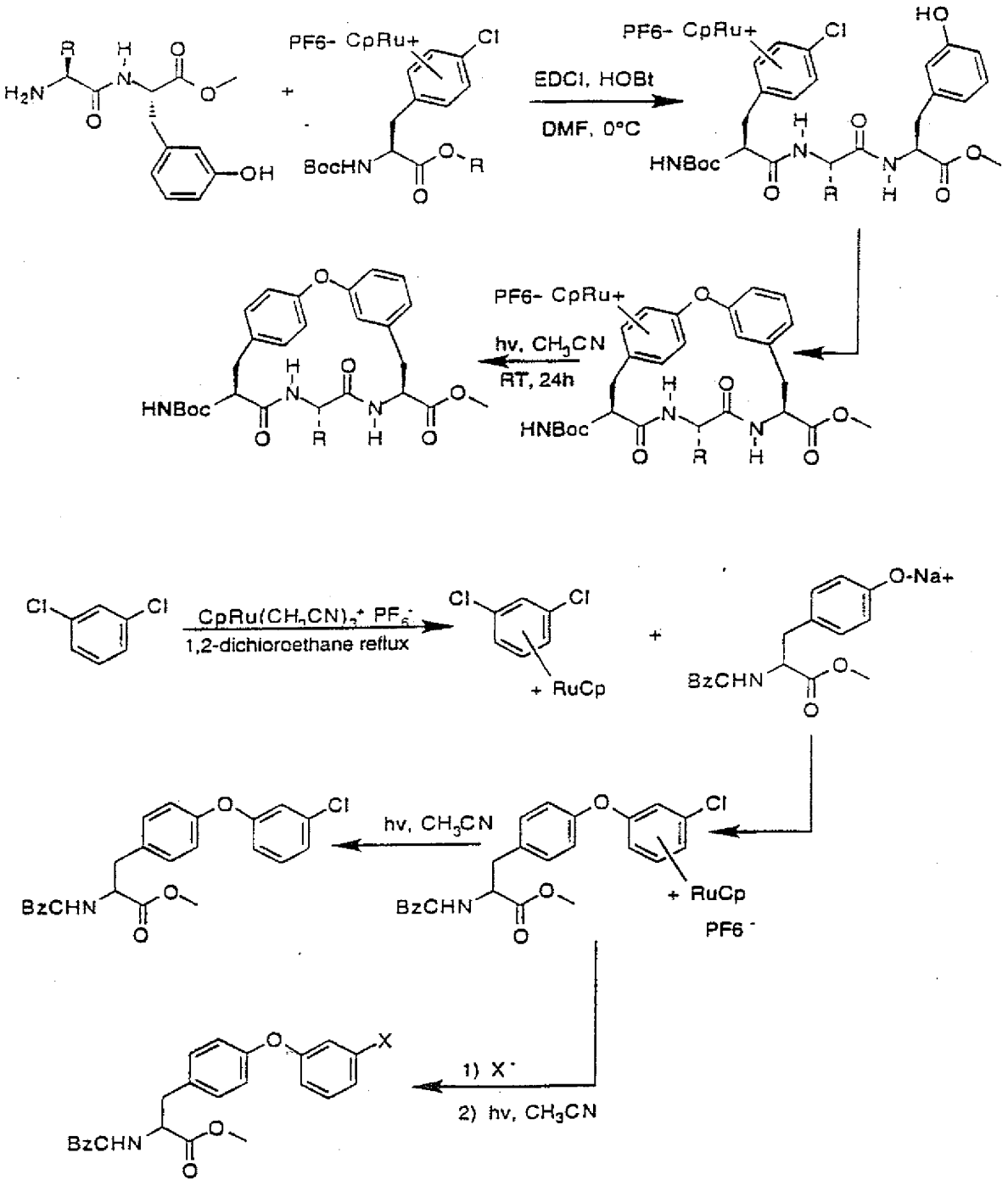
the use of a ruthenium-arene complex that is susceptible to nucleophilic attack such that a chloride substituent is displaced in an S_NAr fashion by, e.g., aryl (or alkyl) alcohols, amines, and sulfides (Scheme I). Pearson, A.J. et al., Studies on Selective Nucleophilic Substitution Reactions of [Cyclopentadienyl)(1,3-dichlorobenzene)M]+PF_6- Complexes (M = Fe, Ru); *J. Org. Chem.* **57**:3583-3589 (1992). Photochemical removal of the Ru catalyst affords the desired compounds and regenerates the catalyst. Zelonka, R.A. et al., Reactions of pi-Benzeneruthenium(II) Complexes with Alkylating Reagents; *J. Organomet. Chem.* **44**:383-389 (1972). This ruthenium method provides aryl ethers with sensitive functional groups and is probably the most general method for making diaryl ethers and selected aryl amines.

In Scheme I, R_1 and R_2 can be a wide variety of functional groups, non-nucleophilic functional groups such as acidic or basic functional groups, or additional halogen groups such as chloro, including protected forms thereof. Phenoxides are more reactive towards the ruthenium π-complexes then are primary amines, anilines or carboxylic acid groups based on the work of Rich and Pearson. Janetka, J.W. et al., Synthesis of Peptidyl Ruthenium p-arene complexes; Application to the synthesis of Cyclic Biphenyl Ether Peptides; *J. Am. Chem. Soc.* **117**:10585-10586 (1995). If necessary, an aryl ether can be prepared with amino and carboxyl groups that are not protected. Sensitive optically pure amino acids can be converted to aryl ethers without compromising stereochemical purity. Janetka, (1995); and Pearson, A.J., et al., Stereoselective synthesis of arylglycine derivatives using Arene-manganese tricarbonyl Complexes; *J. Chem. Soc. Chem. Commun.* (1989)). Scheme II

Scheme I


Cl
RuCp(CH3CN) 3PF6
1,2-DCE, reflux, 1h
85-90%
Boc-HN
CO2R1
1a; R=H
1b; R=Me
CpRu+, PF6−
2a; R=H
2b; R=Me
NaX
R2
hv, CH3CN
RuCp(CH3CN)3PF6
X
3a; X=O
3b; X=NH
3c; X=S

Scheme II and III



illustrates an example of the tolerance of sensitive functionality in this method with the preservation of stereochemistry. To further demonstrate the versatility of this reaction, Scheme III shows the use of dichlorobenzene precursors with the replacement of one or both of the Cl groups to form a variety of molecules (Table I). Pearson, (1992); Moriarty, R.M., et al., Synthesis and nucleophilic Displacement Reactions of Novel η^6-2-chloro-3-methoxyesterone)(η^5-cyclopentadienyl)ruthenium Hexafluorophosphates; *J. Chem. Soc. Chem. Commun.* 1765-1765 (1990)).

TABLE I

X Nucleophile	Nu (conditions)	Typical Yield
MeO-	MeOH-NaH, reflux 5H	90%
C_4H_8N-	pyrrolidine, DMF-THF, 48h	90%
$(MeO_2C)_2CH$-	$(MeO_2)_2CH_2$, NaH, reflux	80%
$(EtO_2C)_2CH$-	$(EtO_2C)_2CH_2$, NaH, reflux	82%
EtNH-	$EtNH_2$, DMF-THF, 48h	76%
OC_4H_8N-	morpholine	95%
L-prolinol-N-	L-prolinol, THF, rt K_2CO_3	99%*
R-ArO-	phenolates, THF	>80%

The above results show that a highly selective sequential nucleophilic displacement of chloride occurs on reaction of 1,3-dichlorobenzene-Ru cations.**

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* Second amine is a better nucleophile than OH.

** (Pearson, A.J., et al., (1992); Moriarty, R.M., et al., (1990); and Pearson, A.J. et al., A Formal Total

Synthesis of the ACE Inhibitor K-13. An application of Arene-Ruthenium Chemistry to Complex Chemical Synthesis; *J. Org. Chem.* **59**, 2304-2313 (1994).

The synthesis of the biphenyl or biaryl compounds can be carried out by the classical Ullmann condensation reaction. For example, the coupling of aryl halides mediated by a Cu^{3+} has been used to prepare a broad range of symmetrical and unsymmetrical biaryls. (Fanta *Chem. Rev.* **64**:613-632 (1964); and Fanta *Synthesis* 9-21 (1974). When a mixture of two different halides are used, one of the three possible products predominantly forms. The best leaving group is iodide, although bromides, chlorides and thiocyanates have been used. Fanta (1964); and Fanta (1974). Compounds with alkyl and etheral links to the aryl substructure can be formed with this method.

While this methodology is generally less preferred for preparing aryls where OH, NHR, NH_2, NHCOR, COOH, SO_2NH_2 functional groups are directly linked to the ring, an alkylene spacer ($-(CH_2)_n-$, where n = 1-8, or similar branched alkylenes) permits synthesis of these compounds. Fanta (1964); and Fanta (1974). Suzuki and Sniekus have developed related strategies for making substituted biaryl systems in a regioselective fashion based on the aryl-aryl cross-coupling strategies. These methods provide biaryl compounds containing multiple sensitive functionalities. (Miyaura, N. et al., Palladium-Catalyzed Cross-Coupling Reaction of Phenyl Boronic Acid with Haloarenes in the Presence of Bases; *Syn. Comm.* **11**:513-519 (1981); Unrau, C.M., et al., Directed Ortho Metalation Suzuki Cross Coupling Connections. Convenient Regiospecific Routes to Functionalized m- and p- teraryls and m- quinquearyls; *Tet. Lett.* **33**, 2772-2776 (1992); Snieckus, V., Directed Ortho Metallation. Tertiary Amide & O-Carbamate Directors in Synthetic Strategies for Polysubstitued Aromatics; *Chem. Rev.* **90**:879-933 (1990); Nesloney, C.L. et al., Synthesis and Hydrogen Bonding

Capabilities of Biphenyl-Based Amino Acids Designed to Nucleate β-Sheet Structure; *J. Org. Chem.* **61**:3127-3137 (1996)). These methods are suitable for preparing a range of biaryls in a regioselective fashion (Schemes IV and V).

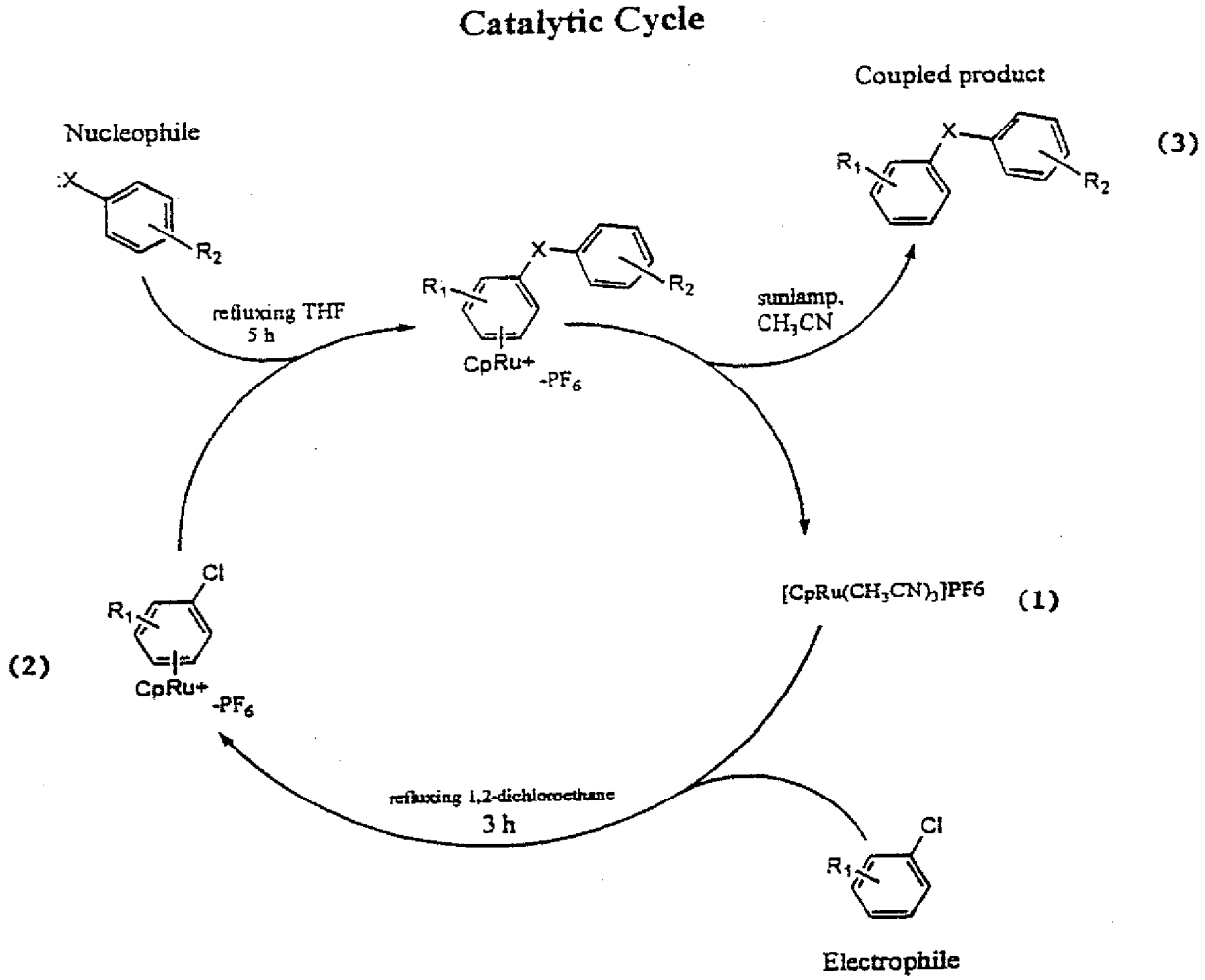
Synthesis of the tertiary amino compounds outlined below can be synthesized with an Ullman condensation between an aniline with an aryl halide and an aniline with a non-aryl R group. Alternatively, alkylation (e.g., with an alkyl halide) of a secondary amine affords the tertiary amine (Scheme VI). A base is not always necessary. As diaryl amines are poor nucleophiles, regarding the alkylene spacer group $-(CH_2)_n-$, both the Ullman and ruthenium strategies work better when n (or m, if any) is 1, 2, or higher, although n = 0 will work also.

Aryl silanes are prepared by the reaction of an aryl anion with a trisubstituted R_3SiCl (where the R's are as described in formula (1a), e.g., alkyl or aryl). Many methods of converting aryl compounds into the conjugate base are known, e.g., subjecting an iodo-substituted aryl to a metal-halogen exchange.

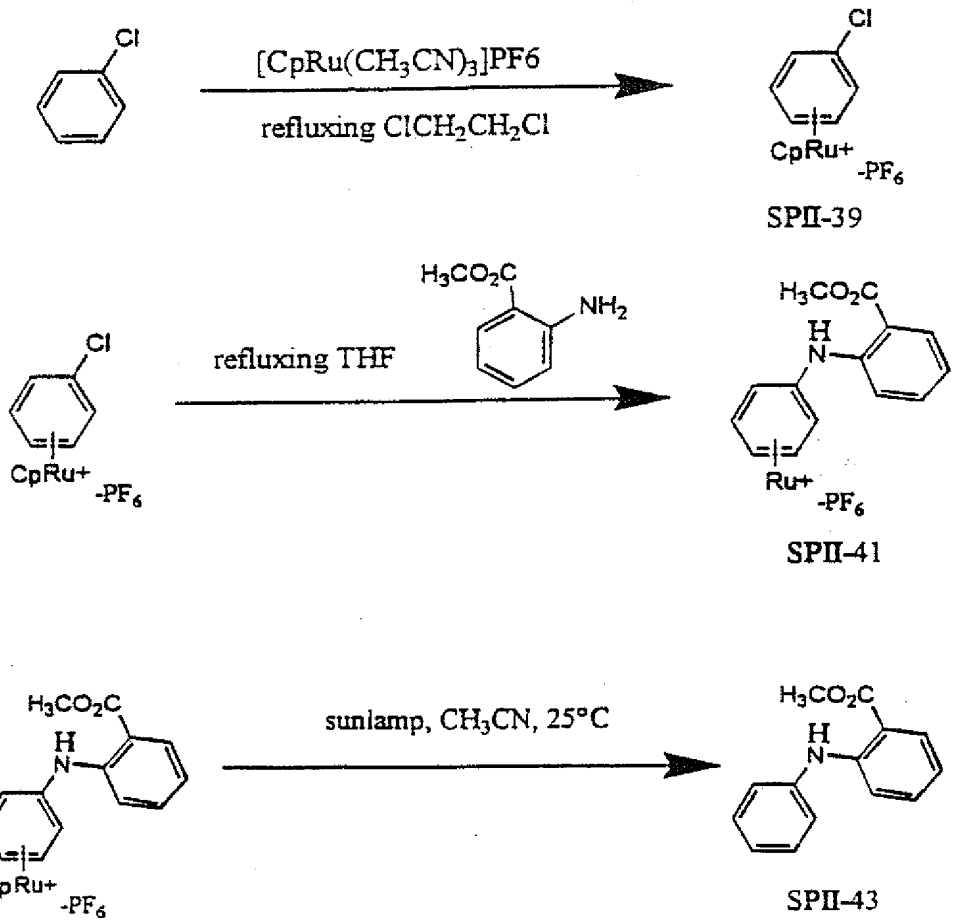
Additional synthetic details are provided in Scheme IV, Scheme V, and Examples 1-4.

Without further elaboration, it is believed that the present invention can be utilized to its fullest extent. The following examples are therefore to be construed as illustrative of the remainder of the disclosure. All publications are hereby incorporated by reference in their entirety.

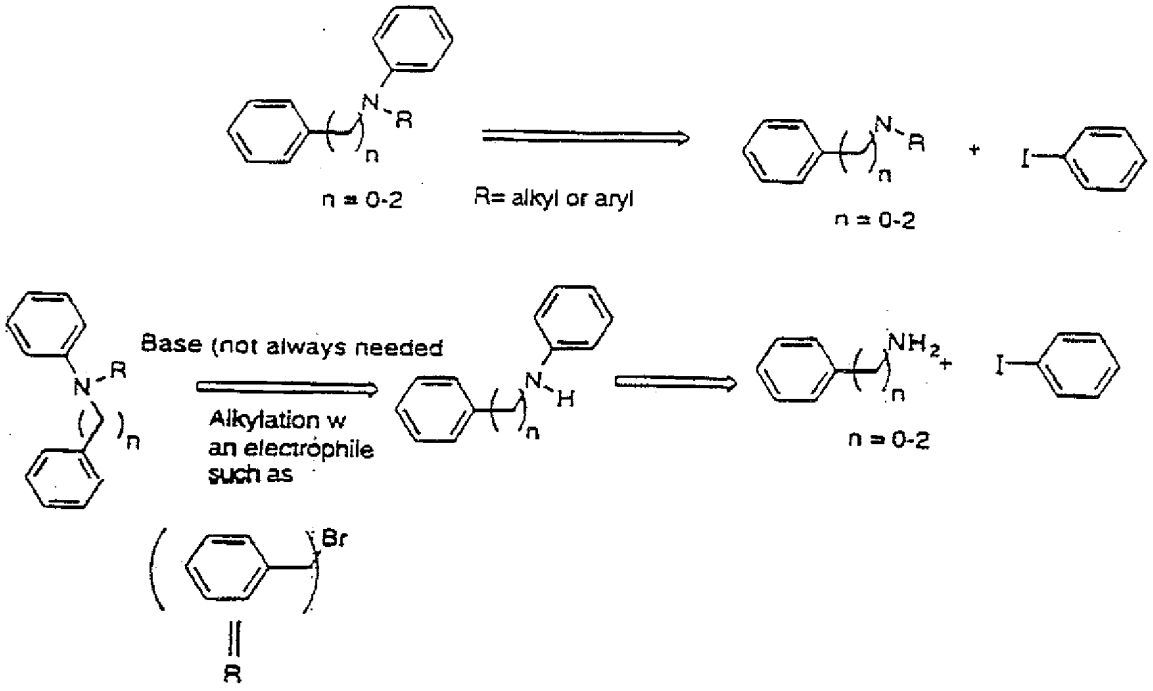
Scheme IV


Catalytic Cycle
Coupled product
(3)
Nucleophile
refluxing THF
5 h
sunlamp.
CH_3CN
$CpRu^+$ $-PF_6$
$[CpRu(CH_3CN)_3]PF6$
(1)
(2)
refluxing 1,2-dichloroethane
3 h
Electrophile

Scheme V


Cl
[CpRu(CH3CN)3]PF6
refluxing ClCH2CH2Cl
CpRu+ -PF6
SPII-39
H3CO2C
NH2
refluxing THF
H3CO2C
H
N
Ru+ -PF6
SPII-41
sunlamp, CH3CN, 25°C
SPII-43

Scheme VI


n = 0-2
R= alkyl or aryl
n = 0-2
Base (not always needed
Alkylation w
an electrophile
such as
Br
R
n = 0-2

Use

The invention provides a method of treating a human amyloid disease, which method includes administering to a patient in need of such treatment an effective amount of a pharmaceutical composition which includes an amyloid stabilizing compound and a pharmaceutically acceptable carrier.

The disclosed compositions can be formulated for oral administration, intravenous injection, topical administration, suppository administration, and implantation, in other words, as solutions, tablets, capsules, or implants, solutions being preferred. Formulations can be prepared for controlled release. The disclosed compositions can be formulated to contain, or can be co-administered with, other amyloid disease drugs, aspirin, anti-inflammatory drugs, and therapeutics which mitigate possible side effects such as altered platelet function, gastro-intestinal erosion, and leukocyte migration. Some embodiments include absorption enhancers.

The attending physician will be able to determine what a pharmaceutically effective dosage would be for a given patient, based on factors such as age, general health, weight, and the extent of the disease or condition to be treated.

Diseases mediated by the abnormal deposition of proteins, such as the formation of fibrils, are treated by administration of compounds which inhibit deposition or fibril formation.

Other Embodiments

From the above description, the essential characteristics of the present invention can be easily ascertained, one of ordinary skill can make various

changes and modifications without departing from the spirit and scope of the invention.

EXAMPLES

Example 1

$(CH_3CN)_3(\eta^5$ -cyclopentadienyl) ruthenium hexafluorophosphate $Ru(III)Cl_3$ hydrate (Aldrich, Milwaukee, WI) was converted into the benzene-ruthenium(II)-dichloride dimer according Baird and Zelontka (cite). Next, Gill and Mann's procedure was used to form the mixed sandwich CpRu (π-benzene)PF_6 salt (Gill and Mann, J. Organometallic 1:485-488 (1982)). This compound was directly converted to the PF_6 salt by precipitation from aqueous hexafluorophosphate. The PF_6 salt was purified by filtration followe by an alumina column with acetone as the eluent to yield pure [CpRu(π -benzene)]PF_6 salt. Photochemistry in bulk acetonitrile with a 400W Ace-Hanovia photoreactor (Ace Glass, Vineland, NJ) gave the labile compound 1 (Scheme V). Over six preparations, a typical procedure involved 3.0g $RuCl_3$ hydrate and yielded 3.0 g [CpRu(CH_3CN)]PF_6 (60% overall yield).

Example 2

(η^6 -Chlorobenzene)η^5 -cyclopentadienyl)ruthenium hexafluorophosphate

To prepare (SPII-39), an oven dried 25 ml round bottomed flask fitted with a condenser was placed under high vacuum, purged with argon four times, and charged with 10 ml of a degassed solution of 1,2-dicholoroethane containing 320 mg of [CpRu(II)(CH_3CH)]PF_6. Next, 100 μl of freshly distilled chloro-benzene was added and the solution was heated to 40°C via an oil bath equipped with a thermowatch. After one hour at 40°C, the slightly brown solution was heated to 70°C for 4 additional hours.

At this point the oil bath was removed and the darker brown solution was allowed to cool to room temperature. Application of mild vacuum gave a dark brownish gum which was placed on high vacuum overnight in the dark. The material was then dissolved in acetone and chromatographed through a short neutral alumina column with acetone as the eluent. ^{1}H NMR (200 MHz, acetone-D_6) ∂ 6.62 (2H, d, J = Hz, Ar), ∂ 6.3 (2H, t, Ar), ∂ 6.18 (1H, d, Ar) ∂ 5.52 (5H, s, Cp); $_{13}$C NMR (75 MHz, acetone-D_6) ∂ 146.9, 116.9, 115.2, 114.7, 111.8.

Example 3

(η^6 -(2- Aminomethylbenzoate)benzene)((η^5 -cyclopentadienyl)ruthenium hexafluorophosphate

After overnight drying under high vacuum, a stir bar was added to 2 which was dissolved with 10 ml of freshly distilled THF. Next, 129 μl of 2-aminomethyl-benzoate was added heat to the stirring solution. The solution was then heated at reflux for 6 hours. This material (compound 2 in Scheme V) was taken on to the next step without further purification.

Example 4

(N-phenyl-2-aminomethylbenzoate, (SPII-43).

In a water-jacketed glass flask compound 2 was dissolved in 250 ml of acetonitrile and the solution was degassed for 30 minutes. After illumination with a 275 W sunlamp for 20 hours, the acetonitrile solution was concentrated *in vacuo* and purified on silica gel flash chromatography (10% EtOAc-hexanes) to give a tan oil (unoptimized). R_f0.45 (35% EtOAc-hexanes); ^{1}H NMR (200 MHz, $CDCl_3$) ∂ 7.72- 7.65 (4H, m, Ar), ∂ 7.57-7.50 (5H, m, Ar), ∂ 3.86 (3H, s, Ar-CO_2CH_3) GC-MS(EI) M^+ = 227, (calc. for $C_{14}H_{13}NO_2$ = 227).

Example 5

Amyloid Fibril Formation and Inhibition via Partial Acid Denaturation

A series of 50 mM sodium acetate-buffered 100 mM KCℓ solutions (1.2 mL) containing different amounts of an amyloidogenic protein-stabilizing compound (e.g., thyroxine or 2,4,6-triiodophenol (TIP)) dissolved in 0.01M NaOH were prepared at the desired pH in microcentrifuge tubes. Wild type TTR from a stock solution (~5 mg/mL TTR in 10 mM phosphate, pH 7.4, 100 mM KCℓ, 1 mM EDTA) was added to each tube to obtain a final TTR concentration of 0.2 mg/mL. Two control samples containing TTR but no amyloidogenic protein-stabilizing compound (thyroxine or TIP), and the compound, but no TTR were also similarly prepared. Each stationary tube was incubated at 37°C for 72 hours to probe the viability of amyloid fibril formation via partial TTR denaturation. The time course of TTR fibril formation under these conditions was reported (see Figure 2B in Lai et al., Biochemistry, 35:6470-82 (1996) demonstrating that amyloid fibril formation plateaus after 72 hours. The extent of fibril formation was measured by optical density (OD) at 330 nm in a standard UV cell, and by a quantitative Congo Red binding assay as described in Lai et al., (1996). Analogous studies were also carried out with L-55-P and V-30-M TTR at pHs where these TTR variants make amyloid (McCutchen et al., Biochemistry, 32:12119-27 (1993); and McCutchen et al., Biochemistry, 34:13527-36 (1995)). The integrity of the amyloid fibrils formed using the methods described above was confirmed by X-ray diffraction, as well as by light and electron microscopy using methods described previously (Colon et al., Biochemistry, 31:8654-60 (1992); and McCutchen et al., (1993).

TTR fibril formation was maximal at pH 4.4, and nearly completely inhibited by the addition of 3 equivalents of T4 (10.8 μM) based on optical density measurements and by quantitative Congo Red binding studies over a 72 hour period. Fibril inhibition decreased with a decrease in the amount of T4 present. Preliminary experiments suggested that ibuprofen and naproxen are not effective inhibitors of amyloid fibril formation.

Inhibition of Amyloid Fibril Formation with Fenoprofen

An evaluation of fenoprofen using the methods described in Example 5 above demonstrated that 10.0 μM fenoprofen effectively binds to transthyretin and prevents the conformational changes required for amyloid fibril formation.

Inhibition of Amyloid Fibril Formation with Flufenamic Acid

An evaluation of flufenamic acid using the methods described in Example 5 above demonstrated that flufenamic acid also inhibits amyloid fibril formation at 10.0 μM.

Example 6

Quaternary Structural Changes: Analytical Ultracentrifugation

The effect of thyroxine on the quaternary structure stability of wild type TTR and single site amyloidogenic variants thereof as a function of pH was evaluated using analytical ultracentrifugation. The quaternary structural changes were examined using sedimentation velocity and sedimentation equilibrium techniques in the presence and absence of an amyloidogenic protein-stabilizing compound (e.g., T4) using a temperature controlled Beckman XL-A analytical ultracentrifuge equipped with a An60Ti rotor and

photoelectric scanner. Double sector aluminum cell centerpieces and quartz windows were used in the velocity experiments at a temperature of 25°C or 37°C, employing 400-420 μL of sample and rotor speeds of 3000-60,000 rpm. Sedimentation of wild type TTR was carried out at pH 4.4 (50 mM acetate, 100 mM KCl). Concentrated TTR stock solutions were spun down on a desk top centrifuge for 15 minutes at 4°C. All buffers were filtered through 0.2μm filter before use. TTR samples (0.2 mg/mL) in the presence or absence of 10.8 μM T4 were prepared as described in the stagnant amyloid fibril formation section. After an incubation period of 48 hours at 25°C or 72 hours at 37°C the samples were loaded into a double sector cell and evaluated at 235 nm. TTR samples lacking T4 incubated at 37°C were not evaluated in the centrifuge due to extensive amyloid fibril formation. For boundary sedimentation analysis, the movement of the midpoint (r) of the absorbance boundary versus time (t) was used to obtain the uncorrected sedimentation coefficient s* from the slope of the plot of ln r -vs-t/ω^2. i.e. ($d\ln r / \omega^2 dt$). The observed sedimentation coefficient, s^*, values obtained were corrected to standard conditions by equation I using tabulated density and viscosity data (Laue et al., Computer aided interpretation of analytical sedimentation data for proteins, in Analytical Ultracentrifugation in Biochemistry and Polymer Science, S.E. Harding, A.J. Rowe, and J.C. Horton, Editors. 1992, Royal Society of Chemistry: Cambridge U.K., p. 90-125) where ρ and η

$$s_{20,w} = S^* \cdot \frac{(\eta)_{T,b}}{(\eta)_{\omega,20}} \cdot \frac{1-\bar{\upsilon}\rho)_{\omega,20}}{(1-\bar{\upsilon}\rho)_{T,b}} \qquad \text{(I)}$$

are the density and viscosity of the solvent at the temperature of the sedimentation velocity experiment or

the viscosity of water at 20°C, and $\bar{\nu}$ is the partial specific volume of the protein under the conditions of the experiment. The sedimentation coefficients $s_{20,w}$ for the TTR tetramer (4.2±0.2S) was determined by sedimentation velocity experiments at pH 7, where the tetramer is the dominant species based on sedimentation equilibrium measurements.

Sedimentation equilibrium runs were performed on 130 μL samples from 3,000-15,000 rpm using double sector cells with charcoal-filled Epon centerpieces and sapphire windows. All scans were performed at 280 nm with a step size of 0.001 and 30 to 50 averages. Samples were allowed to equilibrate over 18-24 hours, and duplicate scans 3 hours apart were overlaid to determine that equilibrium had been reached. The data were analyzed by a nonlinear least square analysis (Johnson et al., Biophysical J., 36:575-88 (1981)) using the Origin™ software provided by Beckman ($\bar{\nu}$ =0.7347 based on amino acid composition). The data were fit to a single ideal species model using equation (II) to determine the best fitting molecular weight.

$$A_r = Exp\ [\ \ln(A_o) + (\ M\omega^2(1-\bar{\upsilon}\rho)/2RT)\cdot(x^2-x^{2o})\ ] + E \qquad \text{(II)}$$

Where A_r is the absorbance at radius x, A_o is the absorbance at a reference radius x_o usually the meniscus, $\overline{\nu\nu}$ is the partial specific volume of TTR (mg/ml), ρ is the density of the solvent (ml/mg), ω is the angular velocity of the rotor (radian/sec), E is the baseline error correction term, M is the gram molecular weight, R is the universal gas constant 8.314 X 10^7 erg/mole, T is temperature (Kelvin).

In the absence of T4 at acidic pHs, TTR formed amyloid and existed in multiple quaternary forms in solution, including the monomeric intermediate.

Example 7

Equilibrium Dialysis Evaluation of Binding to TTR

Equilibrium dialysis was utilized to evaluate the binding stoichiometry of T4 to TTR under acidic conditions with protein and ligand concentrations identical to the concentrations used in Example 5. The top chamber of a dialysis cell, was filled with 2.5 mL of 50 mM acetate buffer containing 0.2 mg/mL [3.63 μM, tetramer] recombinant wild type TTR and a known amount of cold T4 with a trace amount of purified ^{125}I-labelled T4. The bottom chamber was filled with 2.5 mL of acetate buffer only. After 5 days of incubation at 25°C, 200 μL aliquots from each chamber were analyzed in a UKB-Wallac Ria Gamma 1274 Counter to determine the fraction of thyroxine bound. The number of moles T4 bound to TTR was determined by multiplying the fraction of T4 bound by the total T4 concentration divided by the TTR concentration. A Scatchard analysis to determine the binding constant of T4 [1.5 x 10^{-6} - 3 x 10^{-6}M] to TTR [0.05 mg/mL] at pH 4.4 was estimated due to the complications resulting from TTR dissociation at T4 concentrations lower than 1.5 x 10^{-6}M. A Scatchard analysis of the binding of T4 [1 x 10^{-8} - 3 x 10^{-6}M] to TTR at pH 7.4 [0.05 mg/mL] was straightforward.

The data demonstrated that T4 and 2,4,6-triiodophenol were effective inhibitors of TTR amyloid fibril formation *in vitro* at an inhibitor concentration of 10 μM. The physiological concentration of TTR is 0.2 mg/mL (3.63 μM, tetramer).

Example 8

Serum Protein Competitive Binding Assay

Methods of measuring the competitive binding of a disclosed compound to serum binding proteins such as albumin (30-50 mg/ml), transthyretin (0.3 mg.ml), and thyroid binding globulin (0.05 mg/ml) are known to those in the art (Munro et al. *J. Clin. Endocrin. and Metab.* 68:1141-1147 (1989). In one example of a competitive dialysis assay, TTR, TBG, or albumin (0.02 mg/ml) is placed in a dialysis chamber having a 10,000 MW cut-off membrane to evaluate the competitive binding of ^{125}I-T4 and a test compound. As thyroid affinity of each of TTR, TBG, and albumin is known, determination of relative affinity is straightforward. Use of human plasma (serum) depleted in one or more serum proteins can also be used to simulate physiological conditions.

Example 9

TBG Binding Assay

The binding constants of ^{125}I-T4 relative to disclosed amyloidogenic protein-stabilizing compounds to TBG at pH 7.4 is determined. One side of a plexiglass equilibrium dialysis cell is filled with 10 ml of 50 mM phosphate or acetate buffer, 0.1 M KCl, containing 1 μM recombinant TBG; the other side is filled with the same buffer, between 1 x 10^{-9} M to 1 x 10^{-11} M T4 (with a trace amount of ^{125}I-T4), and variable concentrations of the potential amyloidogenic protein-stabilizing compound. After equilibrium is reached (about 48 hours) followed by gamma counting, the data is evaluated with the Scatchard equation to determine the binding constant of the test compound. The K_a for T4 to TBG is 6.3 x 10^{10} L/mol.

Example 10

Thyroid Hormone Receptor Binding Assay

Nuclear thyroid hormone receptors are isolated and partially purified as a group of thyroid receptors as described in J. Biol. Chem. 263:9409-9417 (1988). The binding of 3,3,3'-triiodo-L-thyroxine (T3) and T4 is evaluated in competition with test amyloidogenic protein-stabilizing compounds using a standard competitive Scatchard analysis. The equilibrium dissociation constants of T3 iwth nuclear hormone receptors is about 100-200 pM, indicating a high binding affinity (Spindler et al. J. Biol. Chem. 250:4113-4119 (1975)).

Rapid screening of amyloidogenic protein-stabilizing compounds is conducted, for example, according to the filter binding assay described by Inoue et al. Anal. Biochem. 134:176-183 (1983). Preferably, amyloidogenic protein-stabilizing compounds do not bind strongly to thyroid hormone receptors.

Example 11

X-ray Crystal Structure of TTR-Fenoprofen

TTR can be crystallized by the hanging drop method in the presence of an amyloidogenic protein-stabilizing compound such as fenoprofen (50 μM) at a TTR concentration of 1-30 mg/ml using ammonium sulfate as a precipitant. Crystals can be grown of a TTR-fenoprofen complex that has been solved to 2.5 Å.

Example 12

Other assays for amyloid fibril inhibition

Compounds whose structrues appear to compliment the known binding site of transthyretin can be screened as possible inhibitors using a light scattering assay and a quantitative congo red fibril formation assay. See Colon et al., *Biochemistry* 31:8465-8660 (1992); Lai et

al., *Biochemistry* 35:6470-6482 (1996); Kelly, *Curr. Op. Struct. Biol* 6:11-17 (1966). Specifically, the extent of fibril formation in the presence and absence of inhibitor can be measured by an optical density measurement at 380nm andby a quantitative Congo red binding assay. The inhibition assay is carried out for 72h at 37°C. The TTR (both wild type and mutants such as L55P and V30M can be purfied from an *E. coli* expression system (e.g., McCutchen et al. *Biochemistry*, 32:12119-12127 (1993) and McCutchen et al. *Biochemistry*, 34:13527-13536 (1995)). For example, concentrated stock solutions of flufenamic acid and fenoprofen are prepared by dissolving hte compounds in 0.01mM NaOH. The concentration is obtained by absorbence. A series of 50mM sodium acetate buffered 100mM KCl solutions (1.2ml) containing different concentrations of the compound are prepared. TTR from a 5 mg/mL stoack solution in 10mM phospahte, pH7.4 100mM KCl, 1mM EDTA was then added to obtain a final protein concentration of 0.2mg/ml. Controls were TTR without inhibitor or inhibitor with no TTR. Each tube is incubated at 37°C for 72h. The extent of fibril formation is measured by optical density at 380nm and by a quantitative Congo red binding assay. Colon et al., cited above. Preincubation of the compound (e.g. Flu) with TTR before shifting the pH to amyloid forming conditions exhibits strictly analgous results when the rate for Flu binding to TTR is greater than the denaturation rate to the amyloidogenic intermediate.

Active compounds may be further evaluated by analytical ultracentrifugation to demonstrate that the normal tetrameric structure is adopted in the presence of the inhibitors under acidic conditions that would result in tetramer dissociation and the conformational changes leading to amlyloid formation in the absence of

inhibitor. Miroy et al. *Proc. Nat'l Acad. Sci. USA* 93:15051-15056 (1996); Kelly, *Strucutre* 5:595-600 (1997).

The non-steroidal anti-inflammatory drugs, fenoprofen and flufenamic acid bind to transthyretin, stabilize the non-amyloidogenic tetramer at pH's>4 and inhibit amyloid formation *in vitro*. More specifically, incubation of physiological concentrations of transthyretin at pH 4.4, 37°C (the pH of maximal wild type amyloid fibril formation) resultsn in the conversion of the majority of TTR into amyloid after 72h in the absence, but not in the presence, of 3 eq of flufenamic acid (10.8μM). In the presence of the drug the untracentrifugation data fits nicely to a single species model corresponding to the non-amyloidogenic tetrameric TTR (54kDa). The observed dissociation constant for the $Flu_2 \bullet (TTR)_4$ complex for wild type, V30M and L55P TTR is 148±66nM, 596±383nM and 956±109nM, respectively, as measured by isothermal titration calorimetry, revealing that k_D is lwo enough to saturate TTR when the Flu concentration = 10.8μM, i.e., 3x the phsiological tetramer concentration (3.6μM).

Example 13

TTR-Flu crystal structure

Regarding the crystal structure, co-crystals of flufenamic acid bound to TTR were produced. First crystals of TTR were grown using the hanging drop vapor diffusion method from solutions of 5mg/ml TTR (in 100mM KCl, 100mM phosphate, pH7.4, 1 M ammonium sulfate) equilabrated against 2 M ammonium sulfate. In order to prepare the TTR-drug complex, crystals were soaked for five weeks with a 10-fold molar excess of flufenamic acid to ensure full saturation of both binding sites. The crystal structure of the binary complex was solved using

molecular replacement methods and refined to a resolution of 2.0Å. As in the TTR-T4 complex, two molecules of flufenamic acid were found to bind deeply into the central hormone binding funnels of the TTR tetramer. Flufenamic acid binds into propeller-like conformations which differ in their dihedral angles along the Ar-N-Ar bonds of the drug. The rotation angles ϕ and ϕ' are defined via the atoms C1-C6-N-C1'-C2'; conformation 1: ϕ/ϕ'=-176/39; conformation 2: ϕ/ϕ'=-169/-147.

Due to the two-fold axis along the binding channel of TTR, a statistical disorder model had to be applied. In each binding site four molecules of flufenamic acid with an occupancy of 0.25 have been positioned into their observed density.

The crystallographic data explain both the affinity of the drug towards TTR (able to bind in its low energy solution conformations) as well as the stabilization of the TTR tetramer upon drug binding. In all four binding modes the drug molecule interacts with residues of two adjacent TTR molecules thus mediating several inter-subunit interactions. The CF_3 substituent occupies the innermost halogen binding pocket, interacting with Ser-117, Thr-119, Leu-110 and Ala-108 providing hydrophilic and hydrophobic fluorine contacts (for interaction distances see table II), Figure 1. The biarylamine system of flufenamic acid is placed in a propeller-like conformation into a hydrophobic patch of the T4 binding site between the residues Leu-17 and Ala-108. Finally the carboxylate group on the outer phenyl ring is placed at the entrance of the funnel-shaped binding pocket forming hydrogen bonds with the side chains of two Lys-15 residues.

Binding of flufenamic acid induces a conformational change in TTR, specifically the side

chains of Ser-117 and Thr-119 rearrange to facilitate additional hydrogen bonding between the four subunits, thereby stabilizing the TTR tetramer in addition to what would be expected from simple ligand binding, Figure 2. In the ligand-free tetramer the side chains of all four Ser-117 residues, which are positioned close to the interaction point of the three two-fold axes of the tetramer, are pointing towards the two T4 binding cavities interacting with bulk solvent. Binding of flufenamic acid, however, triggers the side chains of the Ser-117 residues in all four monomers to rotate about 120°, allowing the formation of two non-solvated hydrogen bonds between the Ser-117 residues on adjacent subunits. Furthermore, the induced conformational change of the Thr-119 side chain allows for the formation of a hydrogen bond to an ordered water molecule, which is linked to the carbonyl oxygen of Asp-18 on an adjacent monomer. In summary, Flu binding appears to initiate inter-subunit hydrogen bonding and hydrophobic interactions, the latter being mediated by the arylamine substructure of Flu, which stabilize the quaternary structure of TTR towards pH mediated dissociation and conformation changes associated with amyloidogenesis.

Thus, Flu binds with modest affinity to wild type, L55P and V30M TTR, stabilizing the normally folded state against the pH induced conformational changes that lead to amyloid fibril formation. Since TTR transports only 10-15 % of T4 in plasma (thyroid binding globulin being the main T4 carrier) inhibitor binding to TTR should not perturb thyroid metabolism, especially considering the small effect the TTR knockout has on thyroid metabolism (Palha et al., J. Biol. Chem. 269:33135-33139 (1994).

Example 14

Table II Structure determination.

Intensity data for the apo- and complexed crystal forms of TTR were collected on a DIP2030 imaging plate system (MAC SCIENCE) using a RIGAKU rotating-anode source at 50 kV and 80 mA. The crystals were isomorphous with unit cell dimensions of a = 43.2 Å, b = 85.3 Å, and c = 64.5 Å. They belong to the space group $P2_12_12$, and contain one half of the homo-tetramer in the asymmetric unit. Data were reduced with DENZO and SCALEPACK (Otwinowski, OSCILLATION DATA REDUCTION PROGRAM, PROCEEDINGS OF THE CCP4 STUDY WEEKEND: DATA COLECTION AND PROCESSING (SEERC DARSBURY LABORATORY), 1993) on a Silicon Graphics Indy computer. The protein coordinates from the TTR-bromoflavone complex (Protein Data Bank accession number 1THC) were used as a starting model for the initial refinement of the native structure by molecular dynamics and energy minimization using the program XPLOR (Brunger et al. XPLOR Version 3.1 (Yale U. Press. New Haven CN). Simulated annealing, addition of 56 water molecules and individual temperature factor refinement provided a model which was used to phase a ëcomplex-nativeí difference fourier map. In the resulting map the drug could be located with peaks heights of 10 $_{rms}$ in both binding pockets of the TTR tetramer. A statistical disorder model was applied to account for the twofold crystallographic symmetry of the binding channel. Four molecules of flufenamic acid with half occupancy were positioned into their observed electron density. Four different lowest energy conformations of the drug (with different values for the two C-N-C-C dihedrals) were calculated by the program INSIGHT (BIOSYM INSIGHT II, Molecular Simulations, Inc.) and used as an initial model for the drug in the crystallographic refinement. After a first cycle of simulated annealing using non-strict restraints, 56 water molecules were placed into difference fourier maps.

After another round of slow cooling and subsequent positional and temperature factor refinement the resulting drug conformation was compared against the initial $F_{o,complex} - F_{o,apo}$ maps and unbiased annealed omit maps, phased in absence of the drug. Two conformations of the drug were found to fit the density maps and both forms were included into the refinement applying an alternate conformation model. Because of the lack of interpretable electron densities in the final map, the 9N-terminal and 2 C-terminal residues were not included into the final model. Coordinates have been deposited in the Protein Data Bank, Brookhaven.

In Table III, interaction distances in the complex of TTR/Flu are shown. The values for both binding modes of hte drug have been averaged over the two symmetry related monomers and are listed in Å. Both molecular conformations differe by a rotation along the N-C bonds (conformation 1:ϕ/ϕ' = -169/-147. Residues marked with an asterix are from the symmetry related subunit.

TABLE II

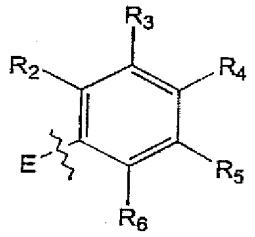
	Apo	TTR*Flu
Resolution (≈)	20-2	20-2
Reflections measured/unique	87447/16489	86716/16664
Completeness (%) overall/outer shell	97.6/79.3	99.6/99.4
R_{sym} (%)* overall/outer shell	6.7/28.9	6.3/28.5
I/σ_I	9.9	10.6
Refinement statistics		
Resolution (≈)**	8-2.2	8-2.0
R_{factor}/R_{free} (%)#∂	18.7/24.5	18.5/24.5
rmsd		
Bond length (≈)	0.014	0.015
Bond angles (degrees)	1.9	2.0

TABLE III

Ligand atom	Protein atom	Distance (Å)	
		Binding mode 1	Binding mode 2
F1	Ser-117 Cβ	3.42	
	Leu-110 N	2.93	
	Leu-110 Cβ	3.07	
	Ser-117 O*		2.66
	Leu-110 N*		2.93
	Leu-110 Cβ*		3.12
F2	Ser-117 Cβ	3.34	
	Leu-110 Cδ2*	3.44	
	Ser-117 Cβ*		3.20
	Leu-110 Cδ2		3.47
F3	Thr-119 Cγ2	3.26	
	Thr-119 N	3.09	
	Ala-108 O	3.01	
	Thr-119 Cγ2*		3.16
	Ala-108 Cβ*		3.75
	Ala-108 O*		3.15
C5'	Leu-110 Cβ	3.93	
C3	Val-121 Cγ2		3.89
	Thr-106 Cγ2	3.92	3.86
C4	Ala-108 Cβ	3.28	3.33
	Val-121 Cγ2	3.89	3.76
	Thr-119 Cγ2	3.68	3.71
	Leu-17 Cδ1*	3.37	3.35
C5	Ala-108 Cβ	3.39	3.20
	Val-17 Cδ1*	2.89	2.98
	Thr-119 Cγ2	3.56	3.53
O1	Lys-15 N	2.98	2.95
	Lys-15 N *	3.59	3.33

CLAIMS

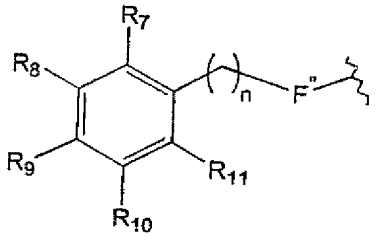
1. A method for treating a human amyloid disease, comprising administering a pharmaceutically effective amount of a composition including an amyloidogenic protein- stabilizing compound; said amyloidogenic protein-stabilizing compound has one of the formulas (1a), (1a'), (1b), or (1c) below:



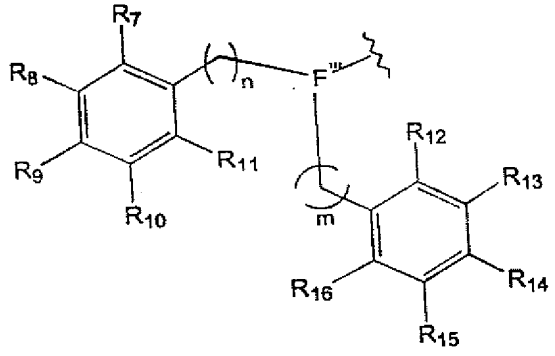
(1a)

wherein E has formula (i), (ii) or (iii):

(i) $-F'-R_{17}$;



(ii)



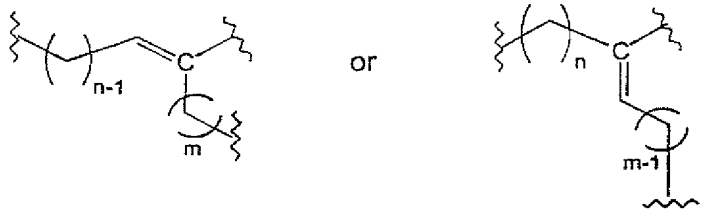
(iii)

in which

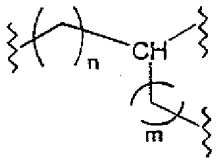
F' is NR_a, O, or S;

F" is NR_a, O, S, CH_2, $Si(R_b)(R_c)$, $-PR_b$;

F''' is N, sp^2 hybridized C as shown in formula (q1) or (q2) or sp^3 hybridized C as shown in formula (q3)



(q1) (q2) ;



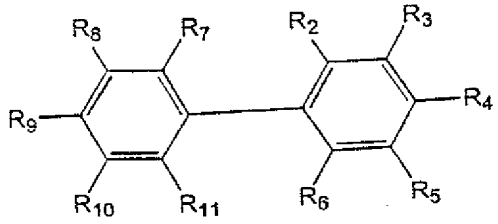
(q3)

and in which:

- R_a is H or C_{1-6} alkyl;
- each of R_b and R_c is independently C_{1-6} alkyl, provided that at least two of R_1 - R_{17} are each independently selected from halo, C_{0-8} hydroxyalkyl, or -A-B, wherein A is branched or straight C_{0-6} alkylene, C_{1-6} acyl, or C_{1-6} aminoalkylene; B is R_d, $-COOR_d$, $-OR_d$, $-SR_d$, $-NHR_d$, or $-N(C_{1-3}$ alkyl$)(R_d)$, R_d being -H, straight or branched C_{1-8} alkyl, C_{2-8} heterocyclic radical, C_{2-8} heteroaryl, or (C_{0-4} alkyl)phenyl; and the remaining R_1 - R_{17} are independently selected from -H, C_{1-6} alkyl, C_{1-6} haloalkyl, benzyl, C_{1-8} alkene, C_{3-8} cycloalkyl, $-N^+R_fR_gR_h$,
- each of R_f, R_g, and R_h being independently selected from -H and C_{1-10} alkyl, (C_{0-4} alkyl)-SO_3H, -(C_{0-4} alkyl)-PO_3H_2, -(C=O)(C_{1-5} alkyl), and $-R_j-M-R_k$, where R_j is straight or branched C_{1-10} alkylene; R_k is H, or straight or branched C_{1-10} alkyl; and M is carbonyl or a heteroatom; or R_j-M-R_k taken together are C_{4-10} cycloalkyl, C_{2-8} heterocyclic radical, or C_{2-8}

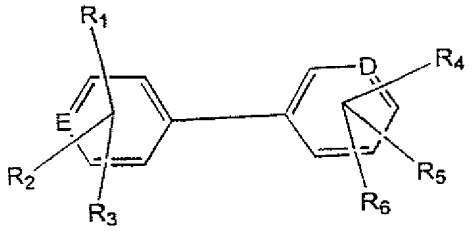
heteroaryl; each of n and m is independently an integer between 0 and 2, inclusive;

(1a'),



in which R_2 - R_{11} are the same as defined above for formual (1a);

(1b)

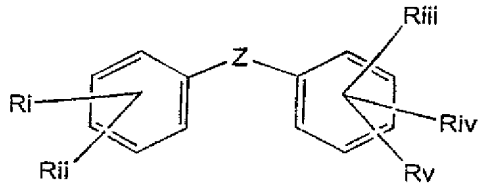


wherein

- each of D and E is independently selected from C, N, O, and S;
- no more than two of R_1 - R_6 are independently selected from -A-B, where A is branched or straight C_{0-6} alkylene, C_{1-6} acyl, or C_{1-6}

aminoalkylene; and B is R_a, $-COOR_a$, $-OR_a$, $-SR_a$, NHR_a, or $N(C_{1-3}$ alkyl$)(R_a)$, R_a being H, straight or branched C_{1-8} alkyl, C_{2-8} heterocyclic radical, C_{2-8} heteroaryl, or $(C_{0-4}$ alkyl$)$phenyl;

- the remaining R_1 - R_6 are independently selected from H, halo, hydroxyl, and cyano;



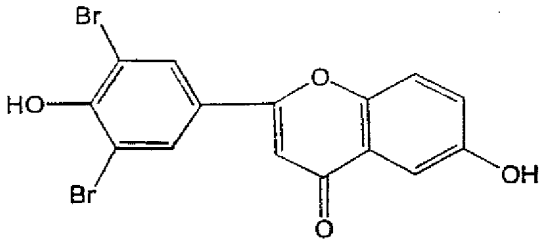
(1c)

wherein

- Z is O or N(G);
- G is selected from H, C_{1-8} alkyl, C_{5-10} cycloalkyl, C_{6-10} aryl, C_{2-8} heterocyclic radical; G being optionally substituted with one or more halo, hydroxy, carboxyl, C_{1-2} haloalkyl, or cyano groups;
- each of R_i - R_v is independently selected from hydrogen, halo, hydroxy, C_{1-5} alkyl, C_{1-5} haloalkyl, cyano, and -A-B as described in formula (1a) above;

2. A method for treating a human amyloid disease, comprising administering a pharmaceutically

effective amount of a composition including an amyloidogenic protein- stabilizing compound; said amyloidogenic protein-stabilizing compound is a nonsteroidal anti-inflammatory compound selected from 2,4,6-triiodophenol, mefenamic acid, diflunisal, meclofenamic acid, milrinone, ethacrynic acid, diclofenac, indomethacin, sulindac, fenclofenac, fenoprofen, flufenamic acid, and EMD 21388 (Merck), (i.e., 2-(3,5-dibromo-4-hydroxyphenyl)-4H-6-hydroxy-1-benzopyran-4-one, see structure below).



3. A method of claim 1 or claim 2, wherein the disease is selected from primary systemic amyloidosis, senile systemic amyloidosis, familial amyloid polyneuropathy I, hereditary cerebral amyloid angiopathy, hemodialysis-related amyloidosis, familial amyloid polyneuropathy III, Finnish hereditary systemic amyloidosis, Type II diabetes, medullary carcinoma of the thyroid, spongiform encephalopathy, atrial amyloidosis, hereditary non-neuropathic systemic amyloidosis, injection-localized amyloidosis, and hereditary renal amyloidosis.

4. A method of claim 3, wherein the disease is senile systemic amyloidosis or familial amyloid polyneuropathy I.

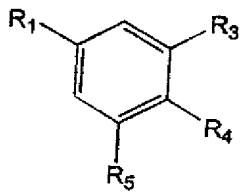
5. A method of claim 1 or claim 2, wherein the disease is Alzheimer's disease.

6. A method of claim 1 or claim 2, wherein the disease is a spongiform encephalopathy.

7. A method of claim 1 or claim 2, wherein the amyloidogenic protein is transthyretin or a variant thereof.

8. A method of claim 1 or claim 2, wherein the compound has the formula (1a).

9. A method of claim 1 or claim 2, wherein the compound has the formula (1d):



(1d)

wherein

- at least one of R_1 and R_4 is selected from F, Cl, Br, I, hydroxy, C_{1-6} haloalkyl (e.g., trifluoromethyl), C_{1-8} alkyl, and -A-B, A being branched or straight C_{0-6} alkylene, C_{1-6} acyl, or C_{1-6} aminoalkylene, and B being R_a, -$COOR_a$, -OR_a, -SR_a, NHR_a, or $N(C_{1-3}$ alkyl$)(R_a)$, R_a being H, straight or branched C_{1-8} alkyl,

C_{2-8} heterocyclic radical, C_{2-8} heteroaryl, or (C_{0-4} alkyl)phenyl;

and

- each of R_3, R_5, R_6, and any remaining R_1 or R_4 is independently selected from H, halo (e.g., I or Br), hydroxyl, C_{2-8} heterocyclic radical, C_{2-8} heteroaryl, and cyano.

10. A method of claim 1, wherein the compound has the formula (1b).

11. A method of claim 1, wherein the compound has the formula (1c).

12. A method of claim 8 wherein the compound is fenoprofen.

13. A method of claim 8, wherein the compound is flufenamic acid.

14. A method of claim 1 or claim 2, wherein the compound binds to transthyretin *in vitro* with a higher affinity than it binds to a human serum binding protein.

15. A method of claim 14, wherein the serum binding protein is thyroxine binding globulin.

16. A method of claim 14, wherein the serum binding protein is serum albumin.

17. A method of claim 1 or claim 2, wherein the compound binds to transthyretin *in vitro* with a higher affinity than it binds to thyroid hormone receptor.

18. An amyloidogenic protein-drug conjugate, comprising an amyloidogenic protein reversibly bound to a compound of formulae (1a), (1a'), (1b), (1c), or (1d).

19. A conjugate of claim 18, wherein the compound is a compound of formula (1c).

20. A conjugate of claim 18, wherein the compound is a biphenylether, a biphenylthioether or a biphenylamine.

21. A conjugate of claim 18, wherein the compound is fenoprofen or flufenamic acid.

22. A method for inhibiting amyloid fibril formation, comprising

forming an amyloidogenic protein/drug conjugate comprising

(a) a compound selected from formula (1a), (1a'), (1b), (1c), and the nonsteroidal anti-inflammatory compounds 2,4,6-triiodophenol, mefenamic acid, diflunisal, meclofenamic acid, milrinone, ethacrynic acid, diclofenac, indomethacin, sulindac, fenclofenac, fenoprofen, flufenamic acid, and EMD 21388 (Merck), (i.e., 2-(3,5-dibromo-4-hydroxyphenyl)-4H-6-hydroxy-1-benzopyran-4-one), or a metabolite thereof (e.g., esters, amines, ammonium salts and Tris salts), and

(b) an amyloidogenic protein reversibly bound to the compound.

23. The method of claim 22, wherein the drug of the amyloidogenic protein/drug conjugate is flufenamic acid.

24. A method for stabilizing an amyloidogenic protein, comprising administering a pharmaceutically effective amount of a composition including an amyloidogenic protein-stabilizing compound having a formula selected from formula (1a), (1a'), (1b), (1c), and the nonsteroidal anti-inflammatory compounds 2,4,6-triiodophenol, mefenamic acid, diflunisal, meclofenamic acid, milrinone, ethacrynic acid, diclofenac, indomethacin, sulindac, fenclofenac, fenoprofen, flufenamic acid and EMD 21388 (Merck), (i.e., 2-(3,5-dibromo-4-hydroxyphenyl)-4H-6-hydroxy-1-benzopyran-4-one), or a metabolite thereof (e.g., esters, amines, ammonium salts and Tris salts).

25. A method of claim 24, wherein the amyloidogenic protein-stabilizing compound is flufenamic acid.

26. A method of claim 24, further comprising the step of forming an amyloidogenic protein/drug conjugate comprising

(a) a compound selected from formula (1a), (1a'), (1b), (1c), and the nonsteroidal anti-inflammatory compounds 2,4,6-triiodophenol, mefenamic acid, diflunisal, meclofenamic acid, milrinone, ethacrynic acid, diclofenac, indomethacin, sulindac, fenclofenac, flufenamic acid, and EMD 21388 (Merck), (i.e., 2-(3,5-dibromo-4-hydroxyphenyl)-4H-6-hydroxy-1-benzopyran-4-one), or a metabolite thereof (e.g., esters, amines, ammonium salts and Tris salts), and

(b) an amyloidogenic protein reversibly bound to said compound.

27. The method of claim 26, wherein the drug of the amyloidogenic protein/drug conjugate is flufenamic acid.

28. A medicament for treating a human amyloid disease comprising the compound of claim 1 or claim 2 in a pharmaceutically acceptable carrier.

29. A method of making a medicament for treating a human amyloid disease comprising mixing the composition of claim 1 or claim 2 with a pharmaceutically acceptable carrier.

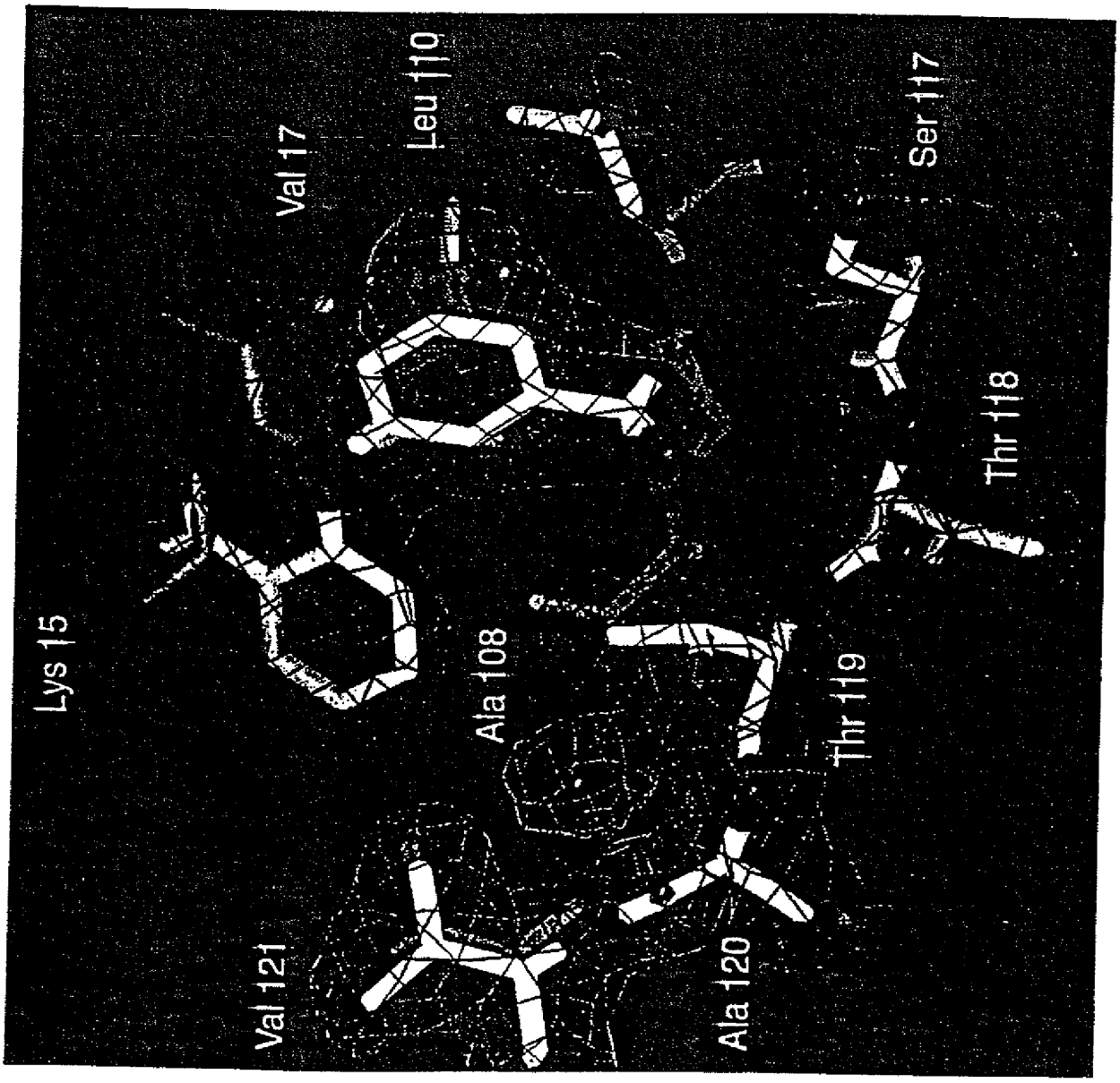

Lys 15
Val 17
Leu 110
Ser 117
Thr 118
Ala 108
Thr 119
Val 121
Ala 120

FIGURE 1

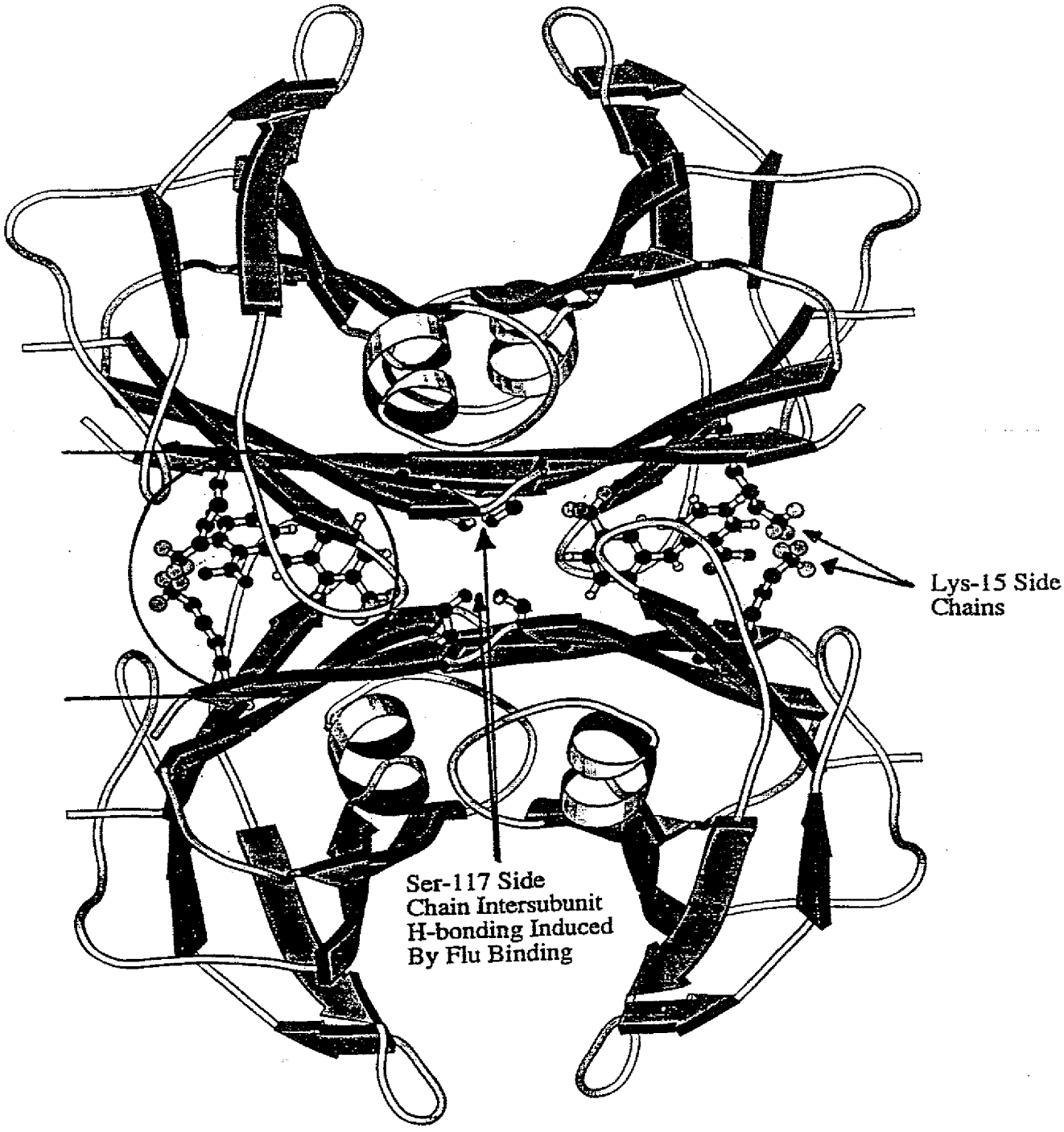

Lys-15 Side Chains
Ser-117 Side Chain Intersubunit H-bonding Induced By Flu Binding

FIGURE 2

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(71) Applicant *(for all designated States except US)*: TEXAS A & M UNIVERSITY [US/US]; 3110 Wisenbaker, College Station, TX 77843 (US).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: DE GUZMAN MIROV, Greta, J. [PH/PH]; 2810 E Zobel Street, Makati City 1200 (PH). KELLY, Jeffery, W. [US/US]; 213 Chimney Hill, College Station, TX 77840 (US). LAI, Zhihong [CN/US]; 3216 Hunter's Glen Drive, Plainsboro, NJ 08536 (US). LASHUEL, Hilal, A. [US/US]; Apartment 6, 198 Court Street, Brooklyn, NY 11201 (US). PETERSON, Scott, A. [US/US]; 3723 Cobblestone Place, Topeka, KS 66610 (US).

(74) Agent: FREEMAN, John, W.; Fish & Richardson P.C., 225 Franklin Street, Boston, MA 02110–2804 (US).

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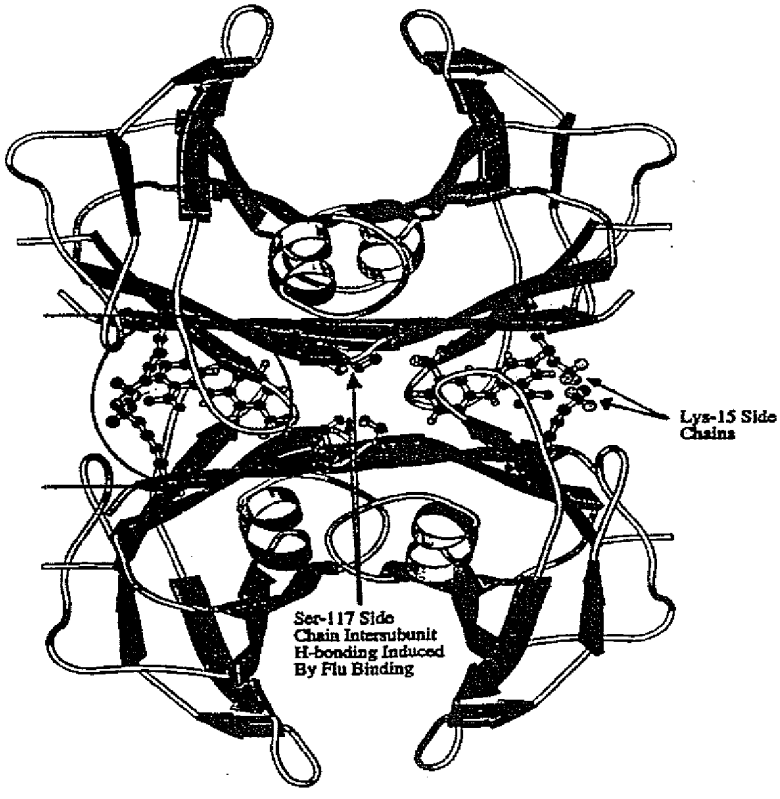
(57) Abstract

A method for treating a human amyloid disease which includes administering a pharmaceutically effective amount of a composition including an amyloidogenic protein–stabilizing aryl compound.



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(75) Inventors/Applicants *(for US only)*: DE GUZMAN MIROV, Greta, J. [PH/PH]; 2810 E Zobel Street, Makati City 1200 (PH). KELLY, Jeffery, W. [US/US]; 213 Chimney Hill, College Station, TX 77840 (US). LAI, Zhihong [CN/US]; 3216 Hunter's Glen Drive, Plainsboro, NJ 08536 (US). LASHUEL, Hilal, A. [US/US]; Apartment 6, 198 Court Street, Brooklyn, NY 11201 (US). PETERSON, Scott, A. [US/US]; 3723 Cobblestone Place, Topeka, KS 66610 (US).

(74) Agent: FREEMAN, John, W.; Fish & Richardson P.C., 225 Franklin Street, Boston, MA 02110–2804 (US).

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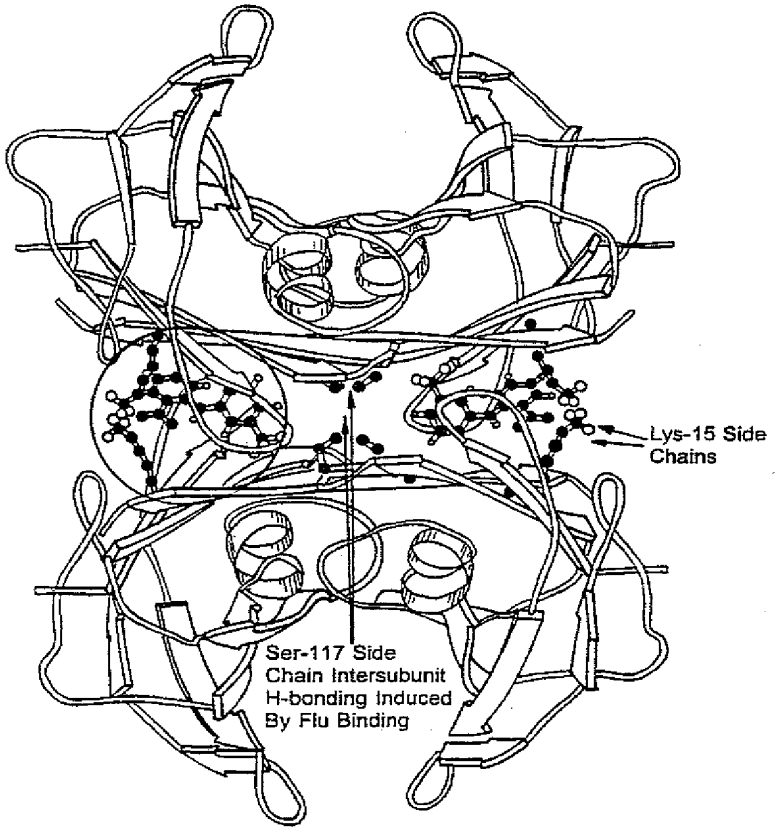
(57) Abstract

A method for treating a human amyloid disease which includes administering a pharmaceutically effective amount of a composition including an amyloidogenic protein–stabilizing aryl compound.



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ANTI-AMYLOIDOGENIC AGENTS

Statement as to Federally Sponsored Research

This invention was made with support from the National Institutes of Health (Grant R29 DK46335-01). Accordingly, the U.S. government may have certain rights in this invention.

Background of the Invention

The invention features anti-amyloidogenic compounds, and methods of using them.

Amyloidosis is characterized by the extracellular deposition in tissue of fibrillar proteins with a β-pleated sheet conformation. Fibrillar proteins are relatively insoluble and resistant to proteolytic digestion.

Transthyretin (TTR), also known as thyroxine binding prealbumin, is found in human plasma as a tetramer of identical 127 residue subunits. Each TTR subunit contains 1 cysteine residue which does not participate in disulfide formation. Each TTR subunit adopts a predominantly β-sheet structure. One four-stranded β-sheet interacts face-to-face with another four-stranded β-sheet, forming a β-sheet sandwich monomer that is the hydrophobic core of the protein. The β-sheet sandwich monomers dimerize through an intermolecular antiparallel β-sheet interaction to form an eight-stranded β-sandwich dimer. Two such dimers form the TTR tetramer.

Normally, tetrameric TTR is the mature fold of the protein. However, in amyloid disease states, tetrameric TTR can be converted into an insoluble fibrillar quaternary structure (an amyloid fibril). The conversion can occur by partial denaturation of the tetramer which

exposes interior residues of each of the four monomers which interact with corresponding residues on other partially-unfolded monomers. Amyloid fibrils are formed from amyloidogenic intermediates under mildly denaturing conditions. An amyloid fibril is an insoluble deposit of an otherwise soluble protein or protein fragments which self-assemble into fibrils about 100 Å in diameter and of variable length. There are at least 16 known human amyloidogenic proteins which have little sequence homology, and yet all are able to form a similar amyloid fibril. Amyloid fibrils may be neurotoxic or may interfere with normal organ function, thereby contributing to a variety of clinical syndromes.

Current therapies for systemic amyloid diseases attempt to remove the source of the precipitating β-protein with drugs that inhibit protein synthesis. In familial amyloid polyneuropathy, for example, a liver transplant is intended to replace the mutant form of TTR produced by the liver, as the mutant form is more readily converted into amyloid in vivo. Current therapies for Alzheimer's disease includes acetylcholinesterase inhibitors which are intended to inhibit the degradation of acetylcholine.

Other diseases associated with amyloid fibril formation are Alzheimer's disease, familial amyooid polyneuropathy (FAP), and senile systemic amyoidosis (SSA).

Summary of the Invention

The invention features a method for treating a human amyloid disease, which method includes administering a pharmaceutically effective amount of a composition including an amyloidogenic protein-stabilizing compound having the formula (1a), (1a'), (1b), (1c), (1d), or the formula of a nonsteroidal anti-

inflammatory agent described in greater detail in the claims.

The disease can be selected from primary systemic amyloidosis, senile systemic amyloidosis, familial amyloid polyneuropathy I, hereditary cerebral amyloid angiopathy, hemodialysis-related amyloidosis, familial amyloid polyneuropathy III, Finnish hereditary systemic amyloidosis, Type II diabetes, medullary carcinoma of the thyroid, spongiform encephalopathy, atrial amyloidosis, hereditary non-neuropathic systemic amyloidosis, injection-localized amyloidosis, and hereditary renal amyloidosis. Preferably the disease is senile systemic amyloidosis or familial amyloid polyneuropathy I; or Alzheimer's disease; or spongiform encephalopathy. In one embodiment, the amyloidogenic protein is transthyretin or a variant thereof.

According to the disclosed method, the compound has the formula (1a), (1a'), (1b), (1c), (1d), or is selected from the individually disclosed compounds. For example, the compound is fenoprofen or flufenamic acid. The invention also features those disclosed compounds which have novel structures.

In one embodiment, an amyloidogenic protein-stabilizing compound binds to transthyretin *in vitro* with a higher affinity than it binds to a human serum binding protein, such as thyroxine binding globulin or serum albumin. The compound may also bind to transthyretin *in vitro* with a higher affinity than it binds to thyroid hormone receptor.

The invention also includes an amyloidogenic protein-drug conjugate. This conjugate includes an amyloidogenic protein reversibly bound to a compound of formulae (1a), (1a'), (1b), (1c), or (1d). Examples of compounds include compounds selected from a

biphenylether, a biphenylthioether or a biphenylamine, such as fenoprofen or flufenamic acid.

The invention also features a method for inhibiting amyloid fibril assembly which includes forming an amyloidogenic protein/drug conjugate, and a method for stabilizing an amyloidogenic protein which includes exposing a target protein to a disclosed compound (e.g., administering a pharmaceutically effective amount of a composition including an amyloidogenic protein-stabilizing compound to a patient, or exposing the target protein *in vitro*). This method for stabilizing an amyloidogenic protein can further include the step of forming an amyloidogenic protein/drug conjugate. Also disclosed are methods for screening for substances which inhibit amyloid fibril assembly by stabilizing an amyloidogenic protein.

Brief Description of the Figures

Fig. 1 is a ball-and-stick model of the flufenamic binding site of TTR. Only residues that line the binding cavity are displayed. Flufenamic acid is shown in one of the two binding modes. Because of the two-fold symmetry along the binding channel two molecules fit the final $2|F_o|-|F_{c\ calc}|$. The CF_3 substituent occupies the innermost T4 hologen binding pocket, interacting with Ser-117, Thr-119, Leu-110 and Ala-108 providing hydrophilic and hydrophobic fluorine contacts. The aromatic rings interact with the hydrophobic patch between the residues Leu-17 and Ala-108. Finally the carboxylate group on the outer phenyl ring is placed at the entrance of hte funnel-shaped binding pocket forming electrostatic interactions with two Lys-15 residues. The image has been generated using the program O (18).

Figure 2 shows that upon binding of flufenamic acid, the side chans of Ser-117, Thr-119, and Lys-15 undergo conformational changes as indicated. The Ser-117

residues particiate in intersubunit hydrogen bonding while the Thr-119 side rotates to form a hydrogen bond to an ordered water molecule which is hydrogen bonded to carbonyl oxygen of Asp-18' on an adjacent subunit.

Detailed Description of the Invention

The invention features a method for treating a protein deposition disease (e.g., an amyloid disease or a light chain deposition disease) which method includes administering a composition which includes an protein assembly inhibitor compound (e.g., an amyloidogenic protein-stabilizing compound), thereby inhibiting protein deposition (e.g., amyloid fibril formation).

Human amyloid diseases, also known as cross-β amyloid fibril-mediated diseases, are defined by the presence of extracellular amyloid deposits which appear to cause disease. Amyloid deposition contributes to neurotoxicity and/or crowds out normal tissue in a given organ, resulting in organ dysfunction. These amyloid diseases can therefore be systemic or neural, and include Alzheimer's disease, primary systemic amyloidosis, senile systemic amyloidosis, familial amyloid polyneuropathy I, hereditary cerebral amyloid angiopathy, hemodialysis-related amyloidosis, familial amyloid polyneuropathy III, Finnish hereditary systemic amyloidosis, Type II diabetes, medullary carcinoma of the thyroid, spongiform encephalopathy, atrial amyloidosis, hereditary non-neuropathic systemic amyloidosis, injection-localized amyloidosis, and hereditary renal amyloidosis. Other diseases include light chain deposition disease (non-amyloid deposits), and prion disease, such as mad cow disease.

Some corresponding amyloidogenic precursor proteins (and fibril components) are, respectively: β protein (β protein 1-40, 1-41, 1-42, and 1-43), immunoglobin light chain (intact light chain or fragments

thereof) (several single site variants), serum amyloid A (amyloid A (76 residue fragment), transthyretin (transthyretin or fragments thereof), transthyretin (over 45 transthyretin variants), cystatin C (cystatin C minus 10 residues), β_2-microglobulin (same), apolipoprotein A-1 (fragments thereof), gelsolin (71 amino acid fragment thereof), Islet amyloid polypeptide or IAPP (fragment of IAPP), calcitonin (fragments thereof), prion (prion or fragments thereof), atrial natriuretic factor or ANF (same), lysozyme (lysozyme or fragments thereof), insulin (same), and fibrinogen (fragments thereof). In some embodiments, the amyloidogenic protein is selected from any of the above, excluding lysozyme, insulin, or both.

An amyloidogenic protein is a precursor protein, or fragment thereof, which can be converted into an insoluble amyloid fibril *in vivo*. Amyloidogenic proteins include a non-fibril conformation, the normally-folded amyloid protein, or a tertiary or quaternary structure that precedes the amyloid such as an oligomer or partially folded protein in the case of a proteolytic fragment of an amyloidogenic protein. Amyloidogenic proteins are generally soluble and include nonnative quaternary structures that precede amyloid fibril assembly, such as a 16-mer, a 44-mer, or a 64-mer. Amyloidogenic proteins also include fibril components (as listed in parentheses above).

An amyloidogenic protein-stabilizing compound can act by binding reversibly, and preferably selectively, to one or more amyloidogenic proteins. Binding to a protein can stabilize an existing protein conformation, or render the protein resistant to the abnormal degradation or partial denaturation which results in amyloid formation or deposition. The binding of the amyloid stabilizing compound to any of the above reduces, in part or whole,

the overall formation of amyloid. Examples of amyloid stabilizing compounds are formulae (1a), (1b), (1c), and (1d). An amyloidogenic protein-stabilizing compound can also act by irreversible binding (covalent linkage) to the amyloidogenic protein.

An amyloidogenic protein-stabilizing compound preferably binds to an amyloidogenic protein, e.g., transthyretin, with a higher affinity that it binds to a non-amyloidogenic protein. In this context, a non-amyloidogenic protein is a protein or receptor, such as a hormone receptor, a human serum binding protein, or an amyloid fibril. A higher affinity refers to a K_d relative to amyloidogenic protein at least 5 times lower (e.g., about one or more magnitudes lower) than the K_d of the non-amyloidogenic protein.

Regarding the higher affinity, it should be noted that TTR liver transplant studies have indicated that TTR amyloid can be cleared *in vivo*. Thus, the combination of the rate of clearance of amyloidogenic proteins (e.g., the quaternary structures such as a 16-mer) and the rate of fibril formation are considered together. Depending on the clinically effective threshold ratio of an amyloidogenic protein/drug conjugate to free amyloidogenic protein, it may be sufficiently effective for an amyloidogenic protein-stabilizing compound to slow down amyloid fibril assembly, rather than substantially stop such assembly. For example, if 20%, 30%, or 40% of the free amyloidogenic protein (e.g., TTR) is in conjugate form, a stabilizing compound having a K_d that is not even one magnitude lower can be effective. An amyloidogenic protein-drug conjugate includes a drug, e.g., an amyloidogenic protein-stabilizing compound reversibly bound to an amyloidogenic protein. Preferably, the reversibly binding is with high affinity (low K_d), and with a low off-rate relative to on-rate so

the drug occupies the amyloidogenic protein for an effective period of time.

Transthyretin includes the wild type protein and any of the more than fifty variants having one or more mutations that are associated with the onset of either senile systemic amyloidosis or familial amyloid polyneuropathy, such as Val-30-Met mutation. Although most single site mutations do not significantly affect the tertiary or quaternary structure of tetrameric TTR, they may nevertheless destabilize the protein. A variant can be used in an *in vitro* assay wherein a disclosed compound binds to TTR with a higher affinity than a serum protein.

Human serum binding proteins include thyroxine binding globulin (TBG), serum albumin, globulins, transferrin, ceruplasmin, glycoproteins, α-lipoproteins, and β-lipoproteins. Thyroid hormone receptors generally regulate growth, development, and metabolic rates in different tissues including cardiovascular, skeletal, gastrointestinal, and neuromuscular systems. Thyroid metabolites of thyroxine such as T3 and agonists or antagonists thereof bind to thyroid hormone receptors. Excessive exposure to thyroid hormone agonists may result in, for example, bradycardia, menstrual abnormalities, weight gain, or anxiety. To avoid undesirable secondary effects, a amyloidogenic protein-stabilizing compound preferably has a relatively low affinity for a thyroid hormone receptors, e.g., binds with a K_d that is at least one order of magnitude, and preferably 1.5, 2, 2.5, or more orders of magnitude higher than the K_d for thyroid metabolites such as T3.

Embodiments

The compounds disclosed herein which inhibit intermolecular aggregation can be used as therapeutics

for any disease or condition that is mediated by an amyloid protein which requires assembly, such as self-assembly of dimeric or oligomeric forms for activity (e.g., neurotoxicity or organ dysfunction). One aspect of the invention therefore features a method of inhibiting amyloid protein assembly, including exposing an aqueous solution of amyloid protein at physiological temperature and pH to an amyloidogenic protein-stabilizing compound. One embodiment further includes measuring the extent of amyloid protein assembly. Turning to an example using a protein precursor such as the oligomeric protein transthyretin (TTR), in one embodiment a preferred compound inhibits TTR fibril formation (Example 7). Sedimentation velocity experiments demonstrated that TTR undergoes conformational change-mediated dissociation to form a monomeric amyloidogenic intermediate which self-assembles into amyloid in the absence of the inhibitor compound (Example 6), but not in its presence (binds to transthyretin, but does not bind in a pharmacologically significant (or adverse) way to thyroxine binding globulin (TBG) (Example 9), a thyroid hormone receptor (i.e., is not a potent thyroxine agonist or antagonist) (Example 10), or other plasma proteins.

Additional *in vitro* assays and animal disease models are known to those in the art, such as those described in Munro, et al., *J. Clin. Endocrinol. and Metab.* 68:1141-1147 (1989) (TTR binding) and Merlini, et al., *Proc. Natl. Acad. Sci.*, 92:2959-2963 (1995) (experimental amyloid murine model, 4'-iodo-4'-deoxydoxorubicin binds to immunoglobulin light chains). Transgenic mice useful for evaluating therapeutics in transthyretin amyloid disease have also been developed by Yamamura et al. *Mol. Biol. Med.* 6:333-343 (1989), and

Buxbaum et al. *Amyloid: Int. J. Exp. Clin. Invest.* 3:187-208 (1996).

Another aspect of the invention features a method of inhibiting intermolecular protein assembly, including exposing a target protein to a disclosed compound. The protein can be in solution, in fibrillar form, or in crystalline form. In one embodiment, the target protein requires monomeric form for function, and is thereby stabilized. In another embodiment, the target protein requires dimeric or tetrameric form for function, and its function is thereby inhibited. In some embodiments, inhibition of protein assembly is useful for storage or analysis of a protein. Similarly, the invention includes a method of inhibiting amyloid protein assembly, comprising exposing an aqueous solution of amyloid protein at physiological temperature to a disclosed peptidomimetic. In some embodiments, the aqueous solution has a pH between 5.0 and 7.5; or the method further includes, after the exposing step, the step of measuring the extent of amyloid protein assembly.

The invention also features a method of down-regulating a target amyloidogenic protein, including administering to a patient an effective amount of a pharmaceutical composition containing containing a disclosed compound. One embodiment of this method reduces the incidence of interaction between the protein and its receptor by interfering with protein-receptor recognition.

Another aspect of the invention is a method of imaging an amyloidogenic protein *in vivo*, including administering a detectable label (e.g., radiolabel or fluorescent label) linked to a disclosed compound, and detecting said label. One embodiment of this method distinguishes between active (e.g., functional or pathological) conformations or assemblies and inactive or

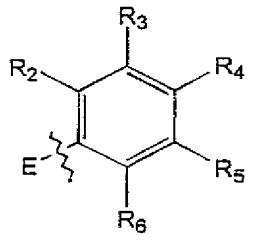
precursor conformations. The link can be a covalent bond or an antibody-antigen interaction.

The invention also includes a method for treating a human light chain deposition disease, comprising administering a pharmaceutically effective amount of a composition including a deposition-inhibiting compound having a formula selected from the formulae (1a), (1a'), (1b), (1c), (1d), and a non-steroidal anti-inflammatory drug.

The invention also features methods of making medicaments for treating human amyloid diseases or conditions characterized, e.g, those by undesired amyloid protein assembly by formulating the designated compounds with a pharmacologically acceptable carrier.

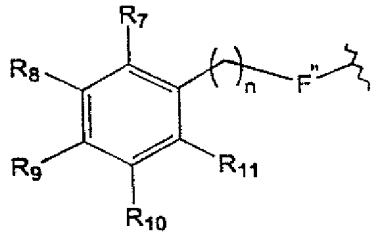
Compounds

The compounds used in the disclosed methods include those of formula (1a):

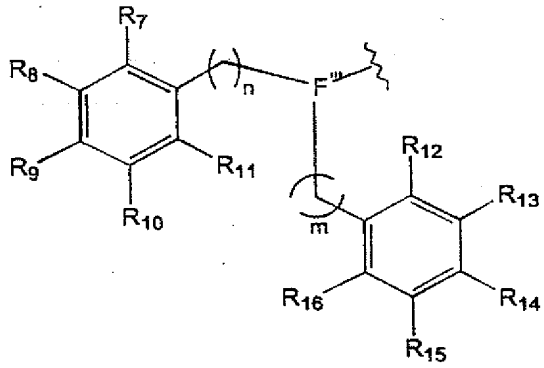


(1a)

wherein E is -F'-R_{17}, formula (i), or formula (ii)



(i)

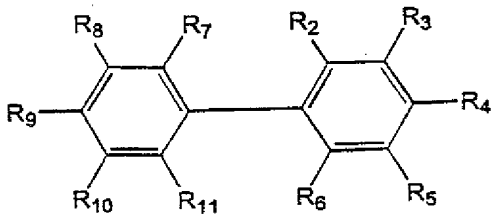


(ii)

in which

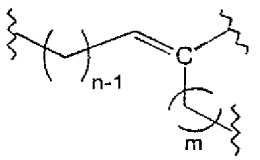
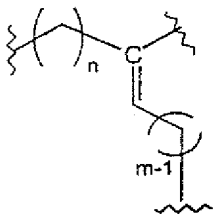
F' is NR_a, O, or S;

F" is NR_a, O, S, CH_2, $Si(R_b)(R_c)$, $-PR_b$, or [-F"-$(CH_2)_n$-] is deleted such that a amyloidogenic protein-stabilizing compound (1a') results;

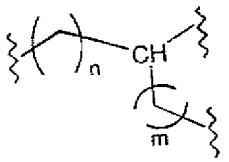


(1a')

F''' is N, sp^2 hybridized C,

i.e.,  or  ,

or sp^3 hybridized C-H,

i.e.,  ;

R_a is H or C_{1-6} alkyl;

each of R_b and R_c is independently C_{1-6} alkyl;

provided that at least two of R_1 - R_{17} are each independently selected from halo, C_{0-8} hydroxyalkyl, or -A-B,

wherein A is branched or straight C_{0-6} alkylene, C_{1-6} acyl, or C_{1-6} aminoalkylene;

B is R_d, $-COOR_d$, $-OR_d$, $-SR_d$, $-NHR_d$, or $-N(C_{1-3}$ alkyl$)(R_d)$;

R_d being H, straight or branched C_{1-8} alkyl, C_{2-8} heterocyclic radical, C_{2-8} heteroaryl, or (C_{0-4} alkyl)phenyl;

and the remaining R_1 - R_{17} are independently selected from H, C_{1-6} alkyl, C_{1-6} haloalkyl, benzyl, C_{1-8} alkene, C_{3-8} cycloalkyl, $N^+R_fR_gR_h$,

each of R_f, R_g, and R_h being independently selected from H and C_{1-10} alkyl, (C_{0-4} alkyl)$-SO_3H$, $-(C_{0-4}$ alkyl$)-PO_3H_2$, $-(C=O)(C_{1-5}$ alkyl), and $-R_j-M-R_k$, where R_j is straight or branched C_{1-10} alkylene; R_k is H, or straight or branched C_{1-10} alkyl; and M is carbonyl or a heteroatom; or R_j-M-R_k taken together are C_{4-10} cycloalkyl, C_{2-8} heterocyclic radical, or C_{2-8} heteroaryl;

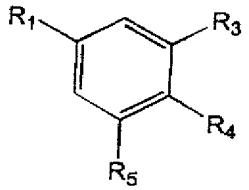
each of n and m is independently an integer between 0 and 2, inclusive (e.g., between 0 and 1). In another aspect, a proximate pair of substituents, such as R_2 and R_7 or R_6 and R_{11}, can be taken together to form a covalent bond.

Representative examples of formula (1a) are shown in Scheme D.

In certain embodiments of formula (1a), there are at least two, at least three, or at least four non-hydrogen ring substituents on each ring or on all rings combined.

In some embodiments, halo is F, Br, or preferably I, e.g., in a mono-iodo or diiodo compound; or there is an acidic group meta or para to the heteroatom (e.g., ether oxygen).

Another compound used in the disclosed methods is a compound of formula (1d)



(1d)

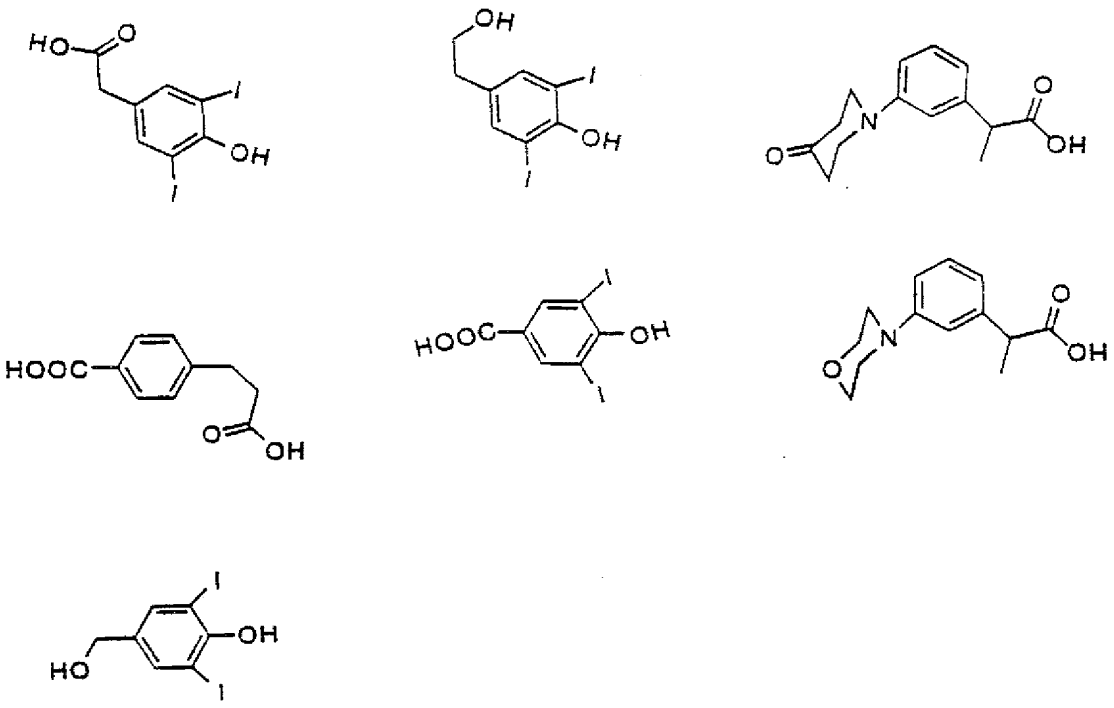
At least one of R_1 and R_4 is selected from F, Cl, Br, I, hydroxy, C_{1-6} haloalkyl (e.g., trifluoromethyl), C_{1-8} alkyl,

-A-B where A is branched or straight C_{0-6} alkylene, C_{1-6} acyl, or C_{1-6} aminoalkylene, and B is R_a, $-COOR_a$, $-OR_a$, $-SR_a$, NHR_a, or $N(C_{1-3}$ alkyl$)(R_a)$, R_a being H, straight or branched C_{1-8} alkyl, C_{2-8} heterocyclic radical, C_{2-8} heteroaryl, or (C_{0-4} alkyl)phenyl. Each of R_3, R_5, R_6, and any remaining R_1 or R_4 is independently selected from H, halo (e.g., I or Br), hydroxy, C_{2-8} heterocyclic radical, C_{2-8} heteroaryl, and cyano. Representative compounds of formula (1d) are shown in Scheme A.

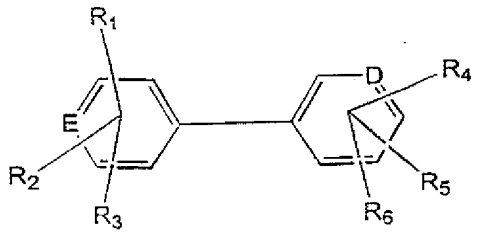
In some embodiments of formula (1d), one of R_1 and R_4 is selected from F, Cl, Br, I, hydroxy, C_{1-6} haloalkyl (e.g., trifluoromethyl), C_{1-8} alkyl, -A-B where A is C_{0-3}

alkylene, C_{1-4} acyl, or C_{1-4} aminoalkylene, and B is R_a, -$COOR_a$, or -OR_a, R_a being H, C_{1-5} alkyl, C_{2-6} heterocyclic radical, or C_{2-5} alkoxyalkyl. R_6 is H and each of R_3, R_5, and any remaining R_1 or R_4 is independently selected from H, I, Br, hydroxy, C_{2-8} heterocyclic radical, and cyano.

Scheme A



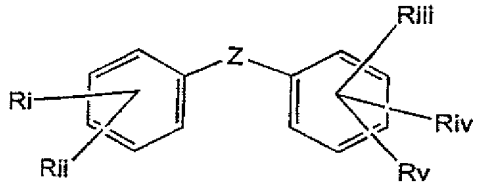
Yet another compound used in the disclosed methods has the formula (1b):



(1b)

Each of D and E is independently selected from C, N, O, and S. No more than two of R_1 - R_6 are independently selected from -A-B where A is branched or straight C_{0-6} alkylene, C_{1-6} acyl, or C_{1-6} aminoalkylene, and B is R_a, -$COOR_a$, -OR_a, -SR_a, NHR_a, or $N(C_{1-3}$ alkyl$)(R_a)$, R_a being H, straight or branched C_{1-8} alkyl, C_{2-8} heterocyclic radical, C_{2-8} heteroaryl, or $(C_{0-4}$ alkyl$)$phenyl. The remaining R_1 - R_6 are independently selected from H, halo, hydroxyl, and cyano. In general, non-hydrogen R_1 - R_6 are preferably in the *meta* or *para* positions relative to the other ring. In some embodiments, electron-withdrawing groups are in one or more of the four available *meta* positions (two on each ring). Representative compounds within formula (1b) are shown in Scheme B.

Other compounds have the formula (1c):

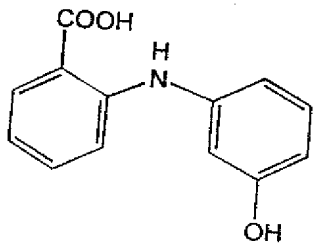


(1c)

Z is O or N(G), where G is selected from H, C_{1-8} alkyl, C_{5-10} cycloalkyl, C_{6-10} aryl, C_{2-8} heterocyclic radical, G being optionally substituted with one or more halo, hydroxy,

carboxyl, C_{1-2} haloalkyl, or cyano groups. Each of R_i - R_v is independently selected from hydrogen, halo, hydroxy, C_{1-5} alkyl, C_{1-5} haloalkyl, cyano, and -A-B as described in formula (1a). In some embodiments, non-hydrogen ring substituents (e.g., electron-withdrawing substituents) are preferably *meta* or *para*; non-hydrogen ring substituents can also be in an *ortho/para*, *ortho/ortho*, or *ortho/para/ortho* arrangement. Representative compounds of formula (1c) are shown in Scheme C.

The invention includes the disclosed method for treating human amyloid diseases using compositions which include known non-steroidal anti-inflammatory drugs (NSAIDs) such as 2,4,6-triiodophenol, mefenamic acid, diflunisal, meclofenamic acid, milrinone, ethacrynic acid, diclofenac, indomethacin, sulindac, fenclofenac, EMD 21388 (Merck), and preferably, fenoprofen and flufenamic acid (Schemes D and E). Flufenamic acid has the following formula:



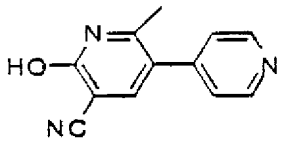
The invention also features metabolic products of disclosed compounds, and prodrugs which, when metabolized, result in a disclosed compound or one or more metabolic products of a disclosed compound. For example, esters, amines, ammonium salts, and Tris salts can be easily formed from the disclosed compounds.

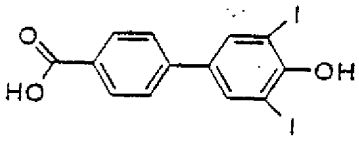
Alkyl groups include straight chain alkyls such as methyl, ethyl, propyl, butyl, and pentyl; branched alkyls

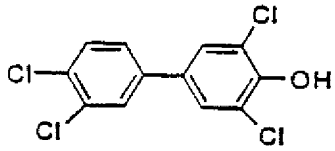
such as isopropyl, isobutyl, t-butyl, sec-pentyl, and 2-methyl-4-ethylheptyl; and cycloalkyls, such as cyclopentyl, cyclohexyl, and 2,4-dimethylcyclohexyl. C_{1-10} alkyls, such as C_{1-6} alkyls or C_{1-3} alkyls.

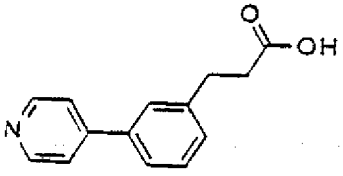
Alkenyl groups are C_{2-10}, e.g., C_{2-8} or C_{2-6}. Like alkyl groups, they include straight chain, branched, and

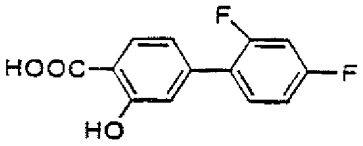
Scheme B

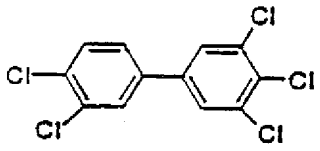


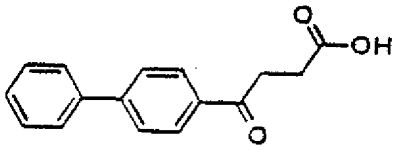




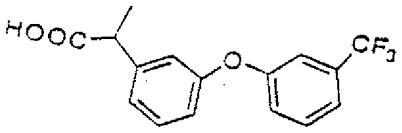


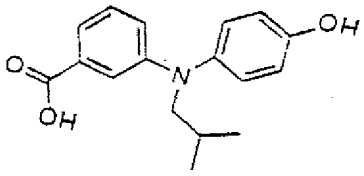


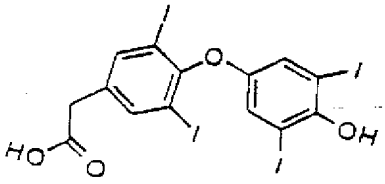


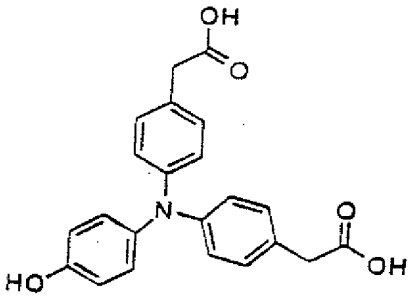


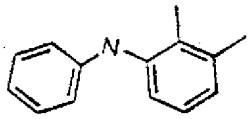
Scheme C

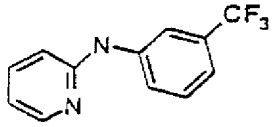

HOOC
O
CF3

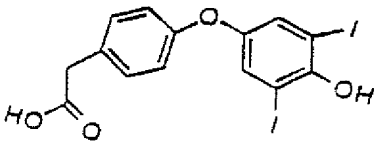

O
OH
N
OH

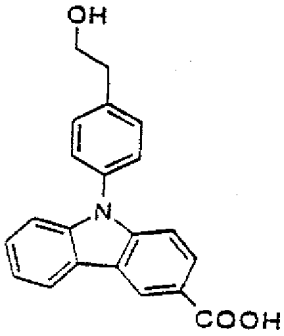

HO
O
I
I
I
O
OH


OH
O
N
HO
O
OH

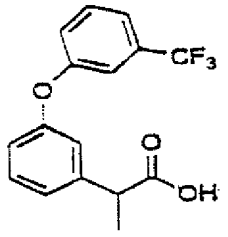

N

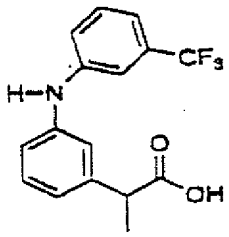

N
CF3
N

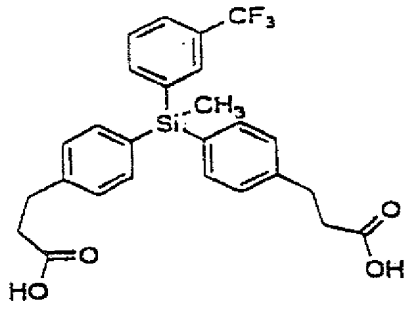

HO
O
O
I
OH
I

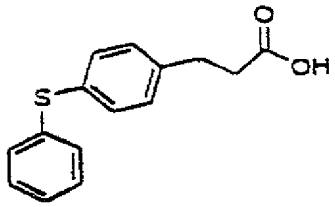

OH
N
COOH

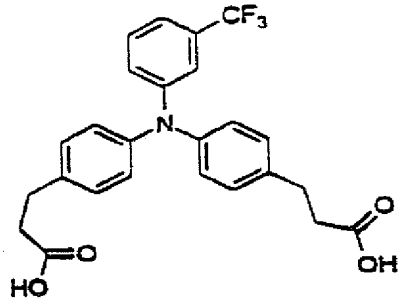
Scheme D

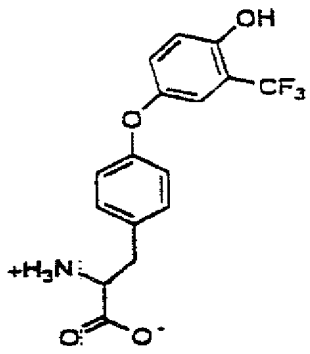

CF3
O
O
OH

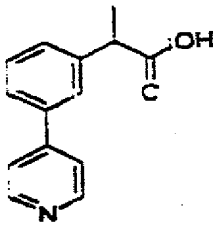

H–N
CF3
O
OH

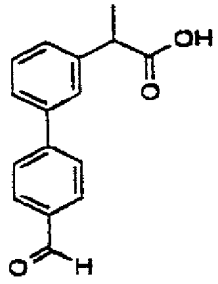

CF3
Si
CH3
O
OH
HO
O

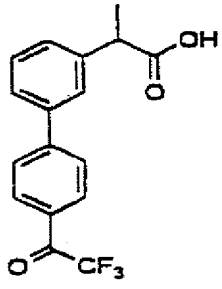

O
OH
S

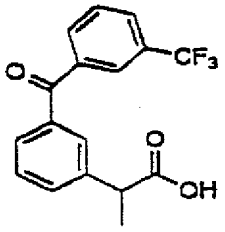

CF3
N
O
OH
HO
O

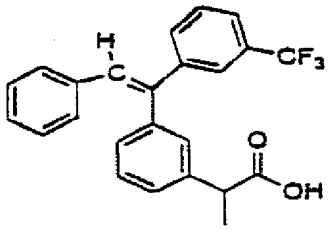

OH
CF3
O
+H3N
O
O-


OH
C
N

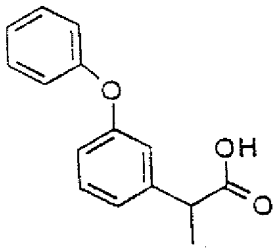

OH
O
O
H


OH
O
O
CF3

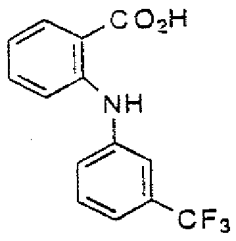

O
CF3
O
OH


H
C
CF3
O
OH

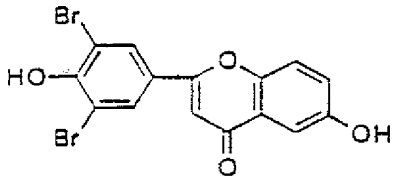
Scheme E



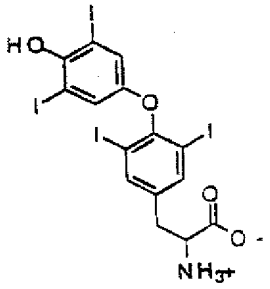
fenoprofen



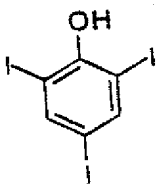
flufenamic acid



EMD 21388



thyroxine



triiodophenol

cyclic moieties. A given double bond may be cis, trans, entgegen, or zusammen. When two or more double bonds are present, they may be conjugated or unconjugated. Alkenyl includes any alkyl group with at least two adjacent hydrogen atoms removed, such as 1-methyl-but-2-enyl, 1,3-butadienyl, isopropenyl, octa-3,6-dienyl, allyl, vinyl, isoprenyl, and prenyl. Alkynyl groups are C_{2-10}, C_{2-8} or C_{2-6}. An alkynyl group may also include one or more double bonds and a triple bond.

Hydroxyalkyl (or hydroxyalkenyl or hydroxyalkynyl) includes any alkyl (or alkenyl or alkynyl) group wherein at least one hydrogen is replaced with a hydroxyl group. Where more than one hydrogen is replaced (e.g., wherein a hydroxy-alkyl is a diol or triol), the hydroxyl groups may be on the same carbon atom (*gem*-diol) or on different carbon atoms (e.g., 1,2-diol or 1,3-diol).

Haloalkyl (or haloalkenyl or haloalkynyl) includes any alkyl (or alkenyl or alkynyl) group wherein at least one hydrogen is replaced with a halogen (fluorine, chlorine, bromine, or iodine). Where more than one hydrogen is replaced (e.g., wherein haloalkyl is a dihaloalkyl or hexahaloalkyl), the halogens are selected independently. For example, halomethyl includes perchloromethyl ($-CF_3$), bromomethyl ($-CH_2Br$), and fluorochloromethyl (-CHFCl). Where more than one hydrogen is replaced, the halogens may be on the same carbon atom (e.g., perfluoroethyl) or on different carbon atoms (e.g., 2-iodo-3-bromobutyl). Amino-substituted and nitro-substituted alkyls (or alkenyls or alkynyls) are analogous to the above.

An aryl group is a C_{6-40} aromatic ring, wherein the ring is made of carbon atoms (e.g., C_{6-20}, C_{6-12}, or C_{6-10} aryl groups). Examples include phenyl, halophenyl, benzyl, naphthyl, binaphthyl, mesityl, tolyl, xylyl, azulyl, indyl, pentalyl, phenanthrenyl, and biphenylyl.

For example, C_8 aryl includes alkylaryls, alkenylaryls, alkynylaryls, arylalkenyls, arylalkynyls, and arylalkyls such as 4-methylbenzyl, 3-ethylphenyl, 4-vinylphenyl (4-ethenylphenyl), and 2,3-dimethylphenyl. (C_6 aryl)(C_4 alkanoyloxy) includes Ph-$(CH_2)_4$(C=O)O- and the (R) and (S) stereoisomers of Ph-$CH_2CH(CH_3)CH_2$(C=O)O-. (C_9 aryl)(C_4 alkanoyloxy) includes (p-ethyl)-Ph$(CH_2)_4$(C=O)O- and the stereoisomers of (2,4-dimethyl)phenyl-$CH_2CH(CH_3)CH_2$(C=O)O-.

A heterocyclic radical contains at least one ring structure which contains carbon atoms and at least one heteroatom such as N, O, or S. A heteroaryl is an aromatic heterocyclic radical. Examples of heterocyclic radicals and heteroaryl groups include: thiazolyl, 2-thienyl, 3-thienyl, 3-furyl, furazanyl, 2H-pyran-3-yl, 1-isobenzofuranyl, 2H-chromen-3-yl, 2H-pyrrolyl, *N*-pyrrolyl, imidazolyl, pyrazolyl, isothiazolyl, isoxazolyl, pyridyl, pyrazinyl, pyrimidinyl, pyridazinyl, indolizinyl, isoindolyl, indolyl, indazolyl, purinyl, phthalazinyl, cinnolinyl, and pteridinyl. For example, a C_{3-12} heteroaryl group may be a C_{3-6}, or a C_{4-9} group. A heterocyclic or heteroaryl radical may be attached to another moiety via a carbon atom or a heteroatom of the heterocyclic or heteroaryl radical.

Aminoalkyls or aminoalkylenes are alkyl or alkylene groups that include substitution with one, two, or more amino (-NH_2) groups. The base alkyl or alkylene can be straight chain or branched, as in thyroxine; an amino group may be charged (e.g., an ammonium salt). Aminoalkyls and aminoalkylenes also include secondary amines substituted with -NHR.

Synthesis

The classical Ullman ether synthesis procedure for making diaryl ethers involves the reaction of an aryloxide with an aryl halide (I≈Br > Cl >> F) (Weingarten, H., Ullman Condensation; *J. Org. Chem.* **29**:977-978 (1964); and Moroz et al., The Ullman Ether Condensation; *Russian Chemical Reviews* **43**:679-689 (1974)) in the presence of a copper salt, typically CuBr, CuBr-SMe_2, or CuCl, in a solvent such as diglyme or xylene. (Lindley, J., Copper Assisted Nucleophilic Substitution of Aryl Halogen; *Tetrahedron* **40**, 1433-1456 (1984)). This method provides simple aryl ethers and will work for preparing those inhibitors that do not contain one or more sensitive functionalities, i.e., a functionality that will decompose in basic solvents in the presence of copper salts at high temperatures (e.g., 160°C) for extended reaction times (e.g., 15 hours). (Moroz, A.A. et al. (1974)).

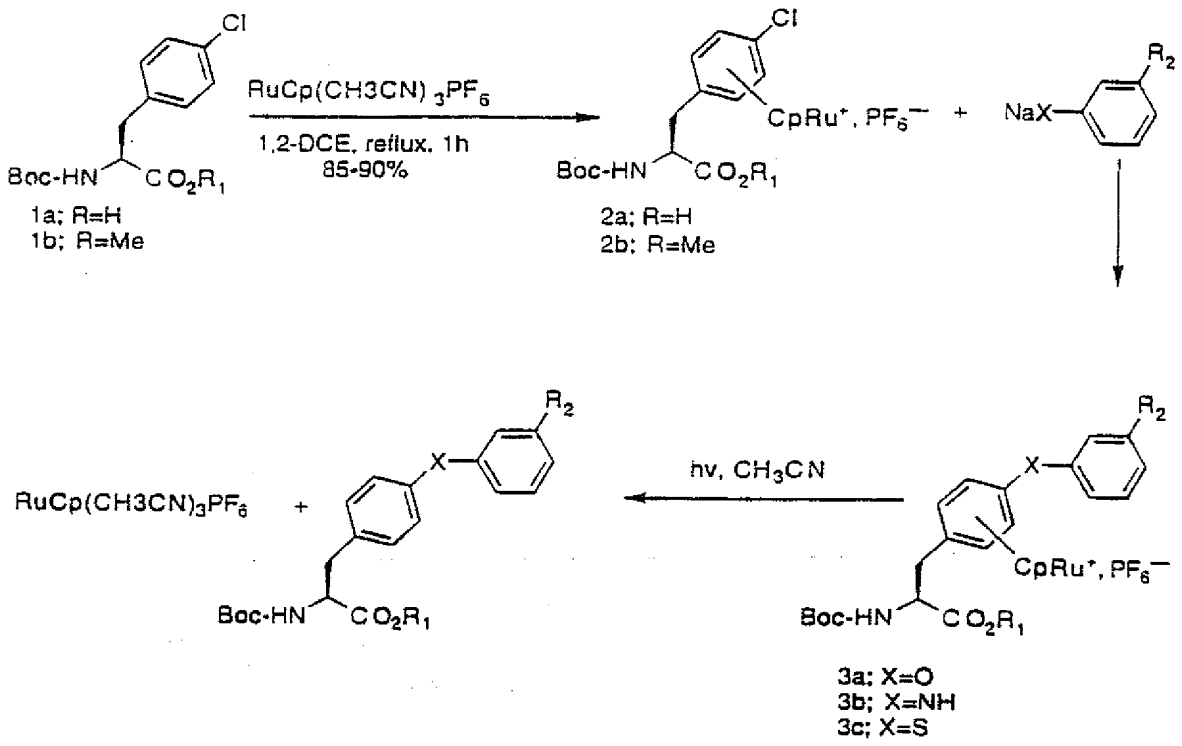
Diarylsulfides can be prepared by reacting the analogous arylsulfide (ArS-) with an aryl halide as described above using polar aprotic solvents. Lindley, (1984). Unactivated aryl halides also give good yields of aryl sulfides on treatment of ArS- in the presence of a catalytic amount of $(Ph_3P)_4Pd$. Diaryl sulfides can also be prepared in high yields by treatment of aryl halides with ArS- in liquid ammonia under irradiation. The Ullman approach also provides aryl amines from a reaction between an aryl halide and a primary amine, a secondary amine, or an aniline. Lindley, (1984). Additional methods for making ethers, thioethers and amines include phenolate displacement of I from aryliodonium salts, and the oxidative phenolic coupling methods of Yamamura and Evans, although these methods are less general.

Newer synthetic methods for the selective production of aryl ethers, sulfides and amines involve

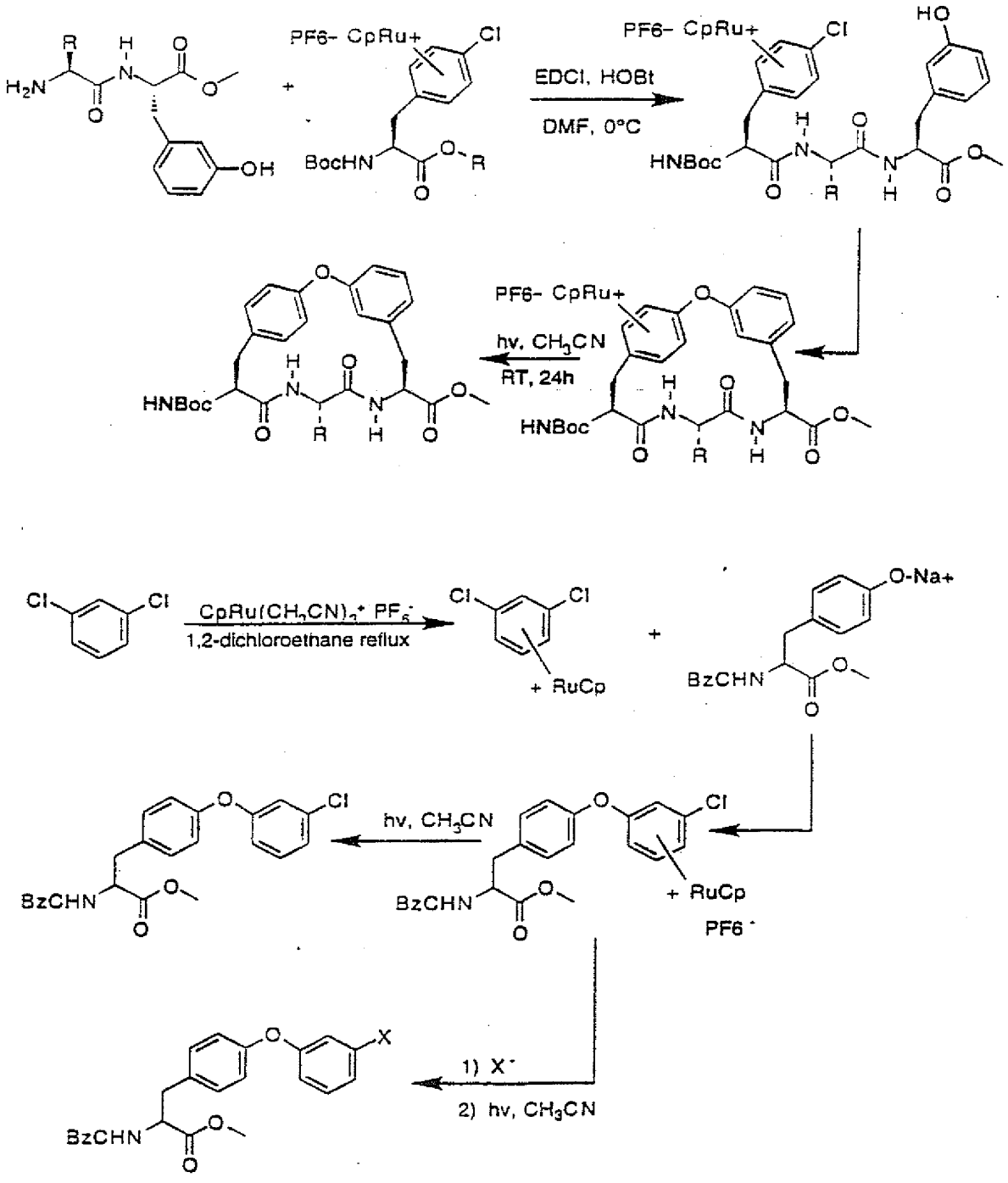
the use of a ruthenium-arene complex that is susceptible to nucleophilic attack such that a chloride substituent is displaced in an S_NAr fashion by, e.g., aryl (or alkyl) alcohols, amines, and sulfides (Scheme I). Pearson, A.J. et al., Studies on Selective Nucleophilic Substitution Reactions of [Cyclopentadienyl)(1,3-dichlorobenzene)M]+PF_6- Complexes (M = Fe, Ru); *J. Org. Chem.* **57**:3583-3589 (1992). Photochemical removal of the Ru catalyst affords the desired compounds and regenerates the catalyst. Zelonka, R.A. et al., Reactions of pi-Benzeneruthenium(II) Complexes with Alkylating Reagents; *J. Organomet. Chem.* **44**:383-389 (1972). This ruthenium method provides aryl ethers with sensitive functional groups and is probably the most general method for making diaryl ethers and selected aryl amines.

In Scheme I, R_1 and R_2 can be a wide variety of functional groups, non-nucleophilic functional groups such as acidic or basic functional groups, or additional halogen groups such as chloro, including protected forms thereof. Phenoxides are more reactive towards the ruthenium π-complexes then are primary amines, anilines or carboxylic acid groups based on the work of Rich and Pearson. Janetka, J.W. et al., Synthesis of Peptidyl Ruthenium p-arene complexes; Application to the synthesis of Cyclic Biphenyl Ether Peptides; *J. Am. Chem. Soc.* **117**:10585-10586 (1995). If necessary, an aryl ether can be prepared with amino and carboxyl groups that are not protected. Sensitive optically pure amino acids can be converted to aryl ethers without compromising stereochemical purity. Janetka, (1995); and Pearson, A.J., et al., Stereoselective synthesis of arylglycine derivatives using Arene-manganese tricarbonyl Complexes; *J. Chem. Soc. Chem. Commun.* (1989)). Scheme II

Scheme I


Cl
RuCp(CH3CN) 3PF6
1,2-DCE, reflux, 1h
85-90%
Boc-HN
CO2R1
1a; R=H
1b; R=Me
CpRu+, PF6−
2a; R=H
2b; R=Me
NaX
R2
X
hv, CH3CN
RuCp(CH3CN)3PF6
3a; X=O
3b; X=NH
3c; X=S

Scheme II and III



illustrates an example of the tolerance of sensitive functionality in this method with the preservation of stereochemistry. To further demonstrate the versatility of this reaction, Scheme III shows the use of dichlorobenzene precursors with the replacement of one or both of the Cl groups to form a variety of molecules (Table I). Pearson, (1992); Moriarty, R.M., et al., Synthesis and nucleophilic Displacement Reactions of Novel η^6-2-chloro-3-methoxyesterone)(η^5-cyclopentadienyl)ruthenium Hexafluorophosphates; *J. Chem. Soc. Chem. Commun.* 1765-1765 (1990)).

TABLE I

X Nucleophile	Nu (conditions)	Typical Yield
MeO-	MeOH-NaH, reflux 5H	90%
C_4H_8N-	pyrrolidine, DMF-THF, 48h	90%
$(MeO_2C)_2CH$-	$(MeO_2)_2CH_2$, NaH, reflux	80%
$(EtO_2C)_2CH$-	$(EtO_2C)_2CH_2$, NaH, reflux	82%
EtNH-	$EtNH_2$,DMF-THF, 48h	76%
OC_4H_8N-	morpholine	95%
L-prolinol-N-	L-prolinol, THF, rt K_2CO_3	99%*
R-ArO-	phenolates, THF	>80%

The above results show that a highly selective sequential nucleophilic displacement of chloride occurs on reaction of 1,3-dichlorobenzene-Ru cations.**

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* Second amine is a better nucleophile than OH.

** (Pearson, A.J., et al., (1992); Moriarty, R.M., et al., (1990); and Pearson, A.J. et al., A Formal Total

Synthesis of the ACE Inhibitor K-13. An application of Arene-Ruthenium Chemistry to Complex Chemical Synthesis; *J. Org. Chem.* **59**, 2304-2313 (1994).

The synthesis of the biphenyl or biaryl compounds can be carried out by the classical Ullmann condensation reaction. For example, the coupling of aryl halides mediated by a Cu^{3+} has been used to prepare a broad range of symmetrical and unsymmetrical biaryls. (Fanta *Chem. Rev.* **64**:613-632 (1964); and Fanta *Synthesis* 9-21 (1974). When a mixture of two different halides are used, one of the three possible products predominantly forms. The best leaving group is iodide, although bromides, chlorides and thiocyanates have been used. Fanta (1964); and Fanta (1974). Compounds with alkyl and etheral links to the aryl substructure can be formed with this method.

While this methodology is generally less preferred for preparing aryls where OH, NHR, NH_2, NHCOR, COOH, SO_2NH_2 functional groups are directly linked to the ring, an alkylene spacer ($-(CH_2)_n-$, where n = 1-8, or similar branched alkylenes) permits synthesis of these compounds. Fanta (1964); and Fanta (1974). Suzuki and Sniekus have developed related strategies for making substituted biaryl systems in a regioselective fashion based on the aryl-aryl cross-coupling strategies. These methods provide biaryl compounds containing multiple sensitive functionalities. (Miyaura, N. et al., Palladium-Catalyzed Cross-Coupling Reaction of Phenyl Boronic Acid with Haloarenes in the Presence of Bases; *Syn. Comm.* **11**:513-519 (1981); Unrau, C.M., et al., Directed Ortho Metalation Suzuki Cross Coupling Connections. Convenient Regiospecific Routes to Functionalized m- and p- teraryls and m- quinquearyls; *Tet. Lett.* **33**, 2772-2776 (1992); Snieckus, V., Directed Ortho Metallation. Tertiary Amide & O-Carbamate Directors in Synthetic Strategies for Polysubstitued Aromatics; *Chem. Rev.* **90**:879-933 (1990); Nesloney, C.L. et al., Synthesis and Hydrogen Bonding

Capabilities of Biphenyl-Based Amino Acids Designed to Nucleate β-Sheet Structure; *J. Org. Chem.* **61**:3127-3137 (1996)). These methods are suitable for preparing a range of biaryls in a regioselective fashion (Schemes IV and V).

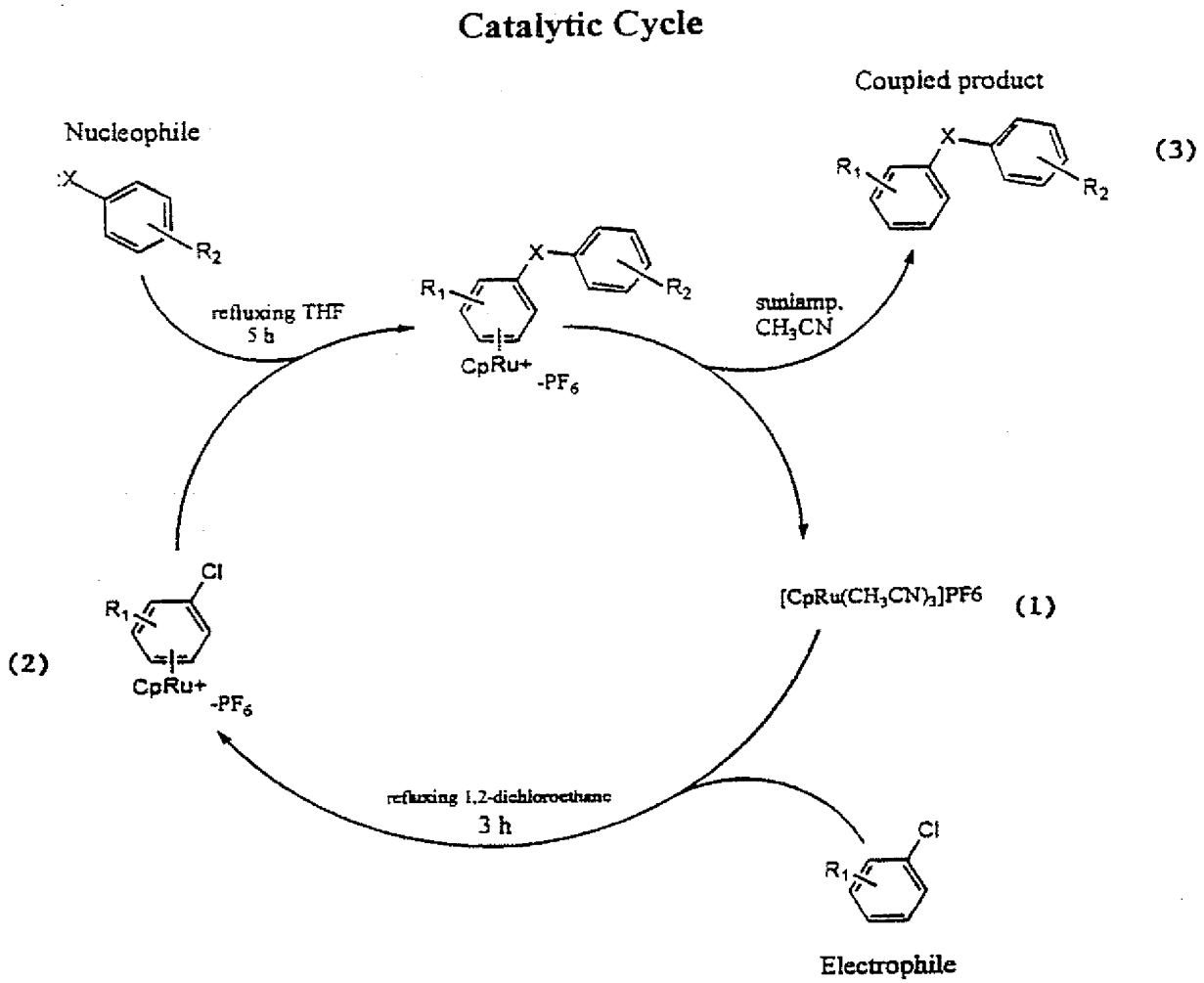
Synthesis of the tertiary amino compounds outlined below can be synthesized with an Ullman condensation between an aniline with an aryl halide and an aniline with a non-aryl R group. Alternatively, alkylation (e.g., with an alkyl halide) of a secondary amine affords the tertiary amine (Scheme VI). A base is not always necessary. As diaryl amines are poor nucleophiles, regarding the alkylene spacer group $-(CH_2)_n-$, both the Ullman and ruthenium strategies work better when n (or m, if any) is 1, 2, or higher, although n = 0 will work also.

Aryl silanes are prepared by the reaction of an aryl anion with a trisubstituted R_3SiCl (where the R's are as described in formula (1a), e.g., alkyl or aryl). Many methods of converting aryl compounds into the conjugate base are known, e.g., subjecting an iodo-substituted aryl to a metal-halogen exchange.

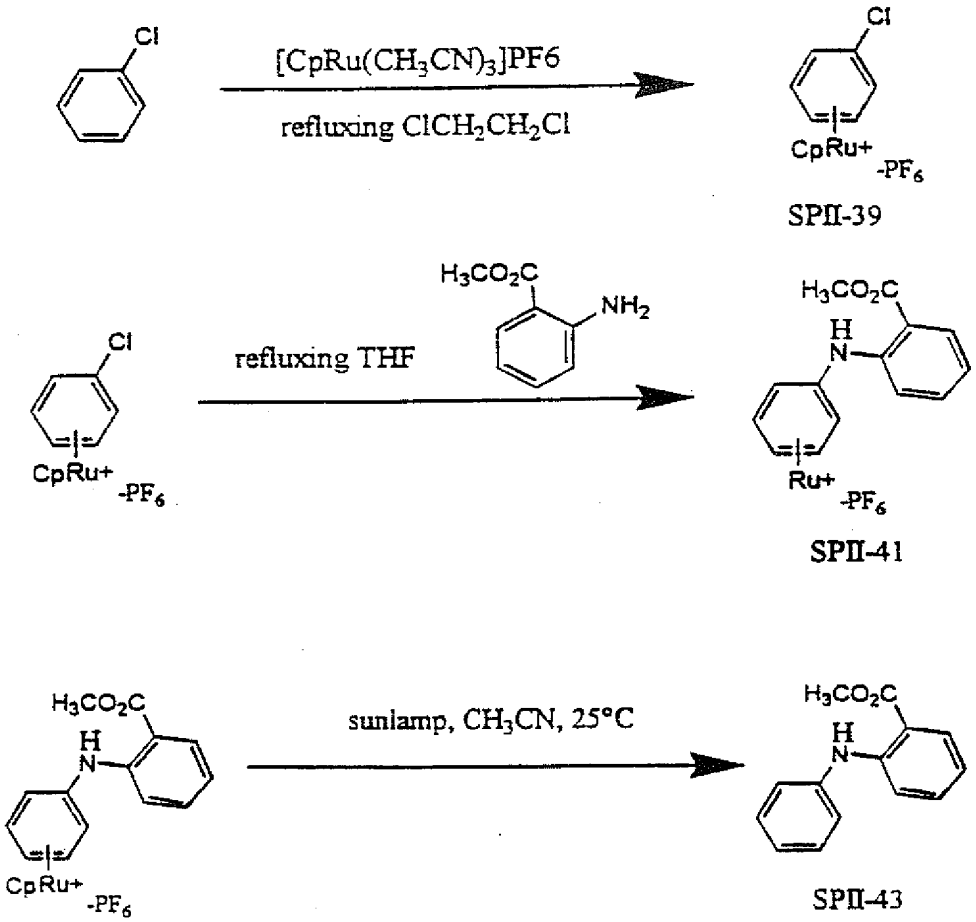
Additional synthetic details are provided in Scheme IV, Scheme V, and Examples 1-4.

Without further elaboration, it is believed that the present invention can be utilized to its fullest extent. The following examples are therefore to be construed as illustrative of the remainder of the disclosure. All publications are hereby incorporated by reference in their entirety.

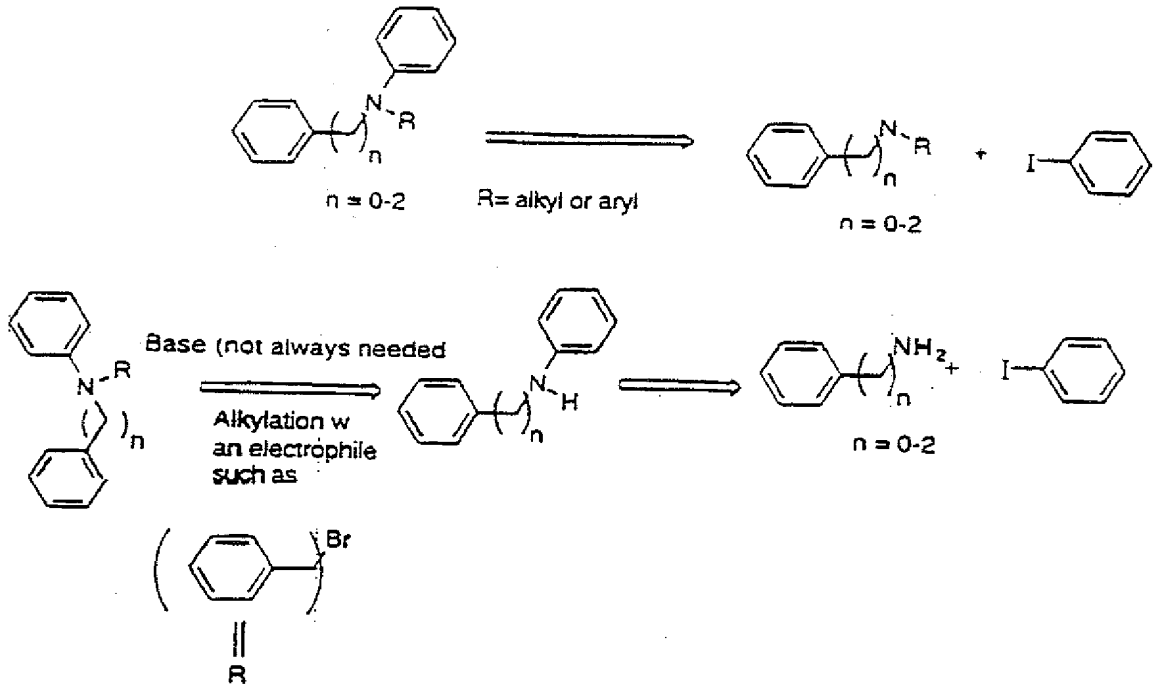
Scheme IV



Scheme V


Cl
[CpRu(CH3CN)3]PF6
refluxing ClCH2CH2Cl
Cl
CpRu+ -PF6
SPII-39
H3CO2C
NH2
refluxing THF
Cl
CpRu+ -PF6
H3CO2C
H
N
Ru+ -PF6
SPII-41
H3CO2C
H
N
CpRu+ -PF6
sunlamp, CH3CN, 25°C
H3CO2C
H
N
SPII-43

Scheme VI


n = 0-2
R= alkyl or aryl
n = 0-2
Base (not always needed
Alkylation w
an electrophile
such as
Br
R
n = 0-2

Use

The invention provides a method of treating a human amyloid disease, which method includes administering to a patient in need of such treatment an effective amount of a pharmaceutical composition which includes an amyloid stabilizing compound and a pharmaceutically acceptable carrier.

The disclosed compositions can be formulated for oral administration, intravenous injection, topical administration, suppository administration, and implantation, in other words, as solutions, tablets, capsules, or implants, solutions being preferred. Formulations can be prepared for controlled release. The disclosed compositions can be formulated to contain, or can be co-administered with, other amyloid disease drugs, aspirin, anti-inflammatory drugs, and therapeutics which mitigate possible side effects such as altered platelet function, gastro-intestinal erosion, and leukocyte migration. Some embodiments include absorption enhancers.

The attending physician will be able to determine what a pharmaceutically effective dosage would be for a given patient, based on factors such as age, general health, weight, and the extent of the disease or condition to be treated.

Diseases mediated by the abnormal deposition of proteins, such as the formation of fibrils, are treated by administration of compounds which inhibit deposition or fibril formation.

Other Embodiments

From the above description, the essential characteristics of the present invention can be easily ascertained, one of ordinary skill can make various

changes and modifications without departing from the spirit and scope of the invention.

EXAMPLES

Example 1

$(CH_3CN)_3(\eta^5$ -cyclopentadienyl) ruthenium hexafluorophosphate $Ru(III)Cl_3$ hydrate (Aldrich, Milwaukee, WI) was converted into the benzene-ruthenium(II)-dichloride dimer according Baird and Zelontka (cite). Next, Gill and Mann's procedure was used to form the mixed sandwich CpRu (π-benzene)PF_6 salt (Gill and Mann, J. Organometallic 1:485-488 (1982)). This compound was directly converted to the PF_6 salt by precipitation from aqueous hexafluorophosphate. The PF_6 salt was purified by filtration followe by an alumina column with acetone as the eluent to yield pure [CpRu(π - benzene)]PF_6 salt. Photochemistry in bulk acetonitrile with a 400W Ace-Hanovia photoreactor (Ace Glass, Vineland, NJ) gave the labile compound 1 (Scheme V). Over six preparations, a typical procedure involved 3.0g $RuCl_3$ hydrate and yielded 3.0 g [CpRu(CH_3CN)]PF_6 (60% overall yield).

Example 2

(η^6 -Chlorobenzene)η^5 -cyclopentadienyl)ruthenium hexafluorophosphate

To prepare (SPII-39), an oven dried 25 ml round bottomed flask fitted with a condenser was placed under high vacuum, purged with argon four times, and charged with 10 ml of a degassed solution of 1,2-dicholoroethane containing 320 mg of [CpRu(II)(CH_3CH)]PF_6. Next, 100 μl of freshly distilled chloro-benzene was added and the solution was heated to 40°C via an oil bath equipped with a thermowatch. After one hour at 40°C, the slightly brown solution was heated to 70°C for 4 additional hours.

At this point the oil bath was removed and the darker brown solution was allowed to cool to room temperature. Application of mild vacuum gave a dark brownish gum which was placed on high vacuum overnight in the dark. The material was then dissolved in acetone and chromatographed through a short neutral alumina column with acetone as the eluent. ^{1}H NMR (200 MHz, acetone-D_6) ∂ 6.62 (2H, d, J = Hz, Ar), ∂ 6.3 (2H, t, Ar), ∂ 6.18 (1H, d, Ar) ∂ 5.52 (5H, s, Cp); $_{13}$C NMR (75 MHz, acetone-D_6) ∂ 146.9, 116.9, 115.2, 114.7, 111.8.

Example 3

(η^6 -(2- Aminomethylbenzoate)benzene)((η^5 -cyclopentadienyl)ruthenium hexafluorophosphate

After overnight drying under high vacuum, a stir bar was added to 2 which was dissolved with 10 ml of freshly distilled THF. Next, 129 μl of 2-aminomethyl-benzoate was added heat to the stirring solution. The solution was then heated at reflux for 6 hours. This material (compound 2 in Scheme V) was taken on to the next step without further purification.

Example 4

(N-phenyl-2-aminomethylbenzoate, (SPII-43).

In a water-jacketed glass flask compound 2 was dissolved in 250 ml of acetonitrile and the solution was degassed for 30 minutes. After illumination with a 275 W sunlamp for 20 hours, the acetonitrile solution was concentrated *in vacuo* and purified on silica gel flash chromatography (10% EtOAc-hexanes) to give a tan oil (unoptimized). R_f0.45 (35% EtOAc-hexanes); ^{1}H NMR (200 MHz, $CDCl_3$) ∂ 7.72- 7.65 (4H, m, Ar), ∂ 7.57-7.50 (5H, m, Ar), ∂ 3.86 (3H, s, Ar-CO_2CH_3) GC-MS(EI) M^+ = 227, (calc. for $C_{14}H_{13}NO_2$ = 227).

Example 5

Amyloid Fibril Formation and Inhibition via Partial Acid Denaturation

A series of 50 mM sodium acetate-buffered 100 mM KCℓ solutions (1.2 mL) containing different amounts of an amyloidogenic protein-stabilizing compound (e.g., thyroxine or 2,4,6-triiodophenol (TIP)) dissolved in 0.01M NaOH were prepared at the desired pH in microcentrifuge tubes. Wild type TTR from a stock solution (~5 mg/mL TTR in 10 mM phosphate, pH 7.4, 100 mM KCℓ, 1 mM EDTA) was added to each tube to obtain a final TTR concentration of 0.2 mg/mL. Two control samples containing TTR but no amyloidogenic protein-stabilizing compound (thyroxine or TIP), and the compound, but no TTR were also similarly prepared. Each stationary tube was incubated at 37°C for 72 hours to probe the viability of amyloid fibril formation via partial TTR denaturation. The time course of TTR fibril formation under these conditions was reported (see Figure 2B in Lai et al., Biochemistry, 35:6470-82 (1996) demonstrating that amyloid fibril formation plateaus after 72 hours. The extent of fibril formation was measured by optical density (OD) at 330 nm in a standard UV cell, and by a quantitative Congo Red binding assay as described in Lai et al., (1996). Analogous studies were also carried out with L-55-P and V-30-M TTR at pHs where these TTR variants make amyloid (McCutchen et al., Biochemistry, 32:12119-27 (1993); and McCutchen et al., Biochemistry, 34:13527-36 (1995)). The integrity of the amyloid fibrils formed using the methods described above was confirmed by X-ray diffraction, as well as by light and electron microscopy using methods described previously (Colon et al., Biochemistry, 31:8654-60 (1992); and McCutchen et al., (1993).

TTR fibril formation was maximal at pH 4.4, and nearly completely inhibited by the addition of 3 equivalents of T4 (10.8 μM) based on optical density measurements and by quantitative Congo Red binding studies over a 72 hour period. Fibril inhibition decreased with a decrease in the amount of T4 present. Preliminary experiments suggested that ibuprofen and naproxen are not effective inhibitors of amyloid fibril formation.

Inhibition of Amyloid Fibril Formation with Fenoprofen

An evaluation of fenoprofen using the methods described in Example 5 above demonstrated that 10.0 μM fenoprofen effectively binds to transthyretin and prevents the conformational changes required for amyloid fibril formation.

Inhibition of Amyloid Fibril Formation with Flufenamic Acid

An evaluation of flufenamic acid using the methods described in Example 5 above demonstrated that flufenamic acid also inhibits amyloid fibril formation at 10.0 μM.

Example 6

Quaternary Structural Changes: Analytical Ultracentrifugation

The effect of thyroxine on the quaternary structure stability of wild type TTR and single site amyloidogenic variants thereof as a function of pH was evaluated using analytical ultracentrifugation. The quaternary structural changes were examined using sedimentation velocity and sedimentation equilibrium techniques in the presence and absence of an amyloidogenic protein-stabilizing compound (e.g., T4) using a temperature controlled Beckman XL-A analytical ultracentrifuge equipped with a An60Ti rotor and

photoelectric scanner. Double sector aluminum cell centerpieces and quartz windows were used in the velocity experiments at a temperature of 25°C or 37°C, employing 400-420 μL of sample and rotor speeds of 3000-60,000 rpm. Sedimentation of wild type TTR was carried out at pH 4.4 (50 mM acetate, 100 mM KCl). Concentrated TTR stock solutions were spun down on a desk top centrifuge for 15 minutes at 4°C. All buffers were filtered through 0.2μm filter before use. TTR samples (0.2 mg/mL) in the presence or absence of 10.8 μM T4 were prepared as described in the stagnant amyloid fibril formation section. After an incubation period of 48 hours at 25°C or 72 hours at 37°C the samples were loaded into a double sector cell and evaluated at 235 nm. TTR samples lacking T4 incubated at 37°C were not evaluated in the centrifuge due to extensive amyloid fibril formation. For boundary sedimentation analysis, the movement of the midpoint (r) of the absorbance boundary versus time (t) was used to obtain the uncorrected sedimentation coefficient s* from the slope of the plot of ln r -vs-t/ω^2. i.e. ($d\ln r / \omega^2 dt$). The observed sedimentation coefficient, s^*, values obtained were corrected to standard conditions by equation I using tabulated density and viscosity data (Laue et al., Computer aided interpretation of analytical sedimentation data for proteins, in Analytical Ultracentrifugation in Biochemistry and Polymer Science, S.E. Harding, A.J. Rowe, and J.C. Horton, Editors. 1992, Royal Society of Chemistry: Cambridge U.K., p. 90-125) where ρ and η

$$s_{20,w} = S^* \cdot \frac{(\eta)T,b}{(\eta)\omega,20} \cdot \frac{1-\bar{v}\rho)\omega,20}{(1-\bar{v}\rho)T,b} \qquad \text{(I)}$$

are the density and viscosity of the solvent at the temperature of the sedimentation velocity experiment or

the viscosity of water at 20°C, and $\bar{\nu}$ is the partial specific volume of the protein under the conditions of the experiment. The sedimentation coefficients $s_{20,w}$ for the TTR tetramer (4.2±0.2S) was determined by sedimentation velocity experiments at pH 7, where the tetramer is the dominant species based on sedimentation equilibrium measurements.

Sedimentation equilibrium runs were performed on 130 μL samples from 3,000-15,000 rpm using double sector cells with charcoal-filled Epon centerpieces and sapphire windows. All scans were performed at 280 nm with a step size of 0.001 and 30 to 50 averages. Samples were allowed to equilibrate over 18-24 hours, and duplicate scans 3 hours apart were overlaid to determine that equilibrium had been reached. The data were analyzed by a nonlinear least square analysis (Johnson et al., Biophysical J., 36:575-88 (1981)) using the Origin™ software provided by Beckman ($\bar{\nu}$ =0.7347 based on amino acid composition). The data were fit to a single ideal species model using equation (II) to determine the best fitting molecular weight.

$$A_r = Exp\ [\ \ln(A_o) + (\ M\omega^2(1-\bar{\upsilon}\rho)/2RT)\cdot(x^2-x^{2o})\ ] + E \qquad \textbf{(II)}$$

Where A_r is the absorbance at radius x, A_o is the absorbance at a reference radius x_o usually the meniscus, $\overline{\nu\nu}$ is the partial specific volume of TTR (mg/ml), ρ is the density of the solvent (ml/mg), ω is the angular velocity of the rotor (radian/sec), E is the baseline error correction term, M is the gram molecular weight, R is the universal gas constant 8.314 X 10^7 erg/mole, T is temperature (Kelvin).

In the absence of T4 at acidic pHs, TTR formed amyloid and existed in multiple quaternary forms in solution, including the monomeric intermediate.

Example 7

Equilibrium Dialysis Evaluation of Binding to TTR

Equilibrium dialysis was utilized to evaluate the binding stoichiometry of T4 to TTR under acidic conditions with protein and ligand concentrations identical to the concentrations used in Example 5. The top chamber of a dialysis cell, was filled with 2.5 mL of 50 mM acetate buffer containing 0.2 mg/mL [3.63 μM, tetramer] recombinant wild type TTR and a known amount of cold T4 with a trace amount of purified ^{125}I-labelled T4. The bottom chamber was filled with 2.5 mL of acetate buffer only. After 5 days of incubation at 25°C, 200 μL aliquots from each chamber were analyzed in a UKB-Wallac Ria Gamma 1274 Counter to determine the fraction of thyroxine bound. The number of moles T4 bound to TTR was determined by multiplying the fraction of T4 bound by the total T4 concentration divided by the TTR concentration. A Scatchard analysis to determine the binding constant of T4 [1.5 x 10^{-6} - 3 x 10^{-6}M] to TTR [0.05 mg/mL] at pH 4.4 was estimated due to the complications resulting from TTR dissociation at T4 concentrations lower than 1.5 x 10^{-6}M. A Scatchard analysis of the binding of T4 [1 x 10^{-8} - 3 x 10^{-6}M] to TTR at pH 7.4 [0.05 mg/mL] was straightforward.

The data demonstrated that T4 and 2,4,6-triiodophenol were effective inhibitors of TTR amyloid fibril formation *in vitro* at an inhibitor concentration of 10 μM. The physiological concentration of TTR is 0.2 mg/mL (3.63 μM, tetramer).

Example 8

Serum Protein Competitive Binding Assay

Methods of measuring the competitive binding of a disclosed compound to serum binding proteins such as albumin (30-50 mg/ml), transthyretin (0.3 mg.ml), and thyroid binding globulin (0.05 mg/ml) are known to those in the art (Munro et al. *J. Clin. Endocrin. and Metab.* 68:1141-1147 (1989). In one example of a competitive dialysis assay, TTR, TBG, or albumin (0.02 mg/ml) is placed in a dialysis chamber having a 10,000 MW cut-off membrane to evaluate the competitive binding of ^{125}I-T4 and a test compound. As thyroid affinity of each of TTR, TBG, and albumin is known, determination of relative affinity is straightforward. Use of human plasma (serum) depleted in one or more serum proteins can also be used to simulate physiological conditions.

Example 9

TBG Binding Assay

The binding constants of ^{125}I-T4 relative to disclosed amyloidogenic protein-stabilizing compounds to TBG at pH 7.4 is determined. One side of a plexiglass equilibrium dialysis cell is filled with 10 ml of 50 mM phosphate or acetate buffer, 0.1 M KCl, containing 1 μM recombinant TBG; the other side is filled with the same buffer, between 1 x 10^{-9} M to 1 x 10^{-11} M T4 (with a trace amount of ^{125}I-T4), and variable concentrations of the potential amyloidogenic protein-stabilizing compound. After equilibrium is reached (about 48 hours) followed by gamma counting, the data is evaluated with the Scatchard equation to determine the binding constant of the test compound. The K_a for T4 to TBG is 6.3 x 10^{10} L/mol.

Example 10

Thyroid Hormone Receptor Binding Assay

Nuclear thyroid hormone receptors are isolated and partially purified as a group of thyroid receptors as described in J. Biol. Chem. 263:9409-9417 (1988). The binding of 3,3,3'-triiodo-L-thyroxine (T3) and T4 is evaluated in competition with test amyloidogenic protein-stabilizing compounds using a standard competitive Scatchard analysis. The equilibrium dissociation constants of T3 iwth nuclear hormone receptors is about 100-200 pM, indicating a high binding affinity (Spindler et al. J. Biol. Chem. 250:4113-4119 (1975)).

Rapid screening of amyloidogenic protein-stabilizing compounds is conducted, for example, according to the filter binding assay described by Inoue et al. Anal. Biochem. 134:176-183 (1983). Preferably, amyloidogenic protein-stabilizing compounds do not bind strongly to thyroid hormone receptors.

Example 11

X-ray Crystal Structure of TTR-Fenoprofen

TTR can be crystallized by the hanging drop method in the presence of an amyloidogenic protein-stabilizing compound such as fenoprofen (50 μM) at a TTR concentration of 1-30 mg/ml using ammonium sulfate as a precipitant. Crystals can be grown of a TTR-fenoprofen complex that has been solved to 2.5 Å.

Example 12

Other assays for amyloid fibril inhibition

Compounds whose structrues appear to compliment the known binding site of transthyretin can be screened as possible inhibitors using a light scattering assay and a quantitative congo red fibril formation assay. See Colon et al., *Biochemistry* 31:8465-8660 (1992); Lai et

al., *Biochemistry* 35:6470-6482 (1996); Kelly, *Curr. Op. Struct. Biol* 6:11-17 (1966). Specifically, the extent of fibril formation in the presence and absence of inhibitor can be measured by an optical density measurement at 380nm andby a quantitative Congo red binding assay. The inhibition assay is carried out for 72h at 37°C. The TTR (both wild type and mutants such as L55P and V30M can be purfied from an *E. coli* expression system (e.g., McCutchen et al. *Biochemistry*, 32:12119-12127 (1993) and McCutchen et al. *Biochemistry*, 34:13527-13536 (1995)). For example, concentrated stock solutions of flufenamic acid and fenoprofen are prepared by dissolving hte compounds in 0.01mM NaOH. The concentration is obtained by absorbence. A series of 50mM sodium acetate buffered 100mM KCl solutions (1.2ml) containing different concentrations of the compound are prepared. TTR from a 5 mg/mL stoack solution in 10mM phospahte, pH7.4 100mM KCl, 1mM EDTA was then added to obtain a final protein concentration of 0.2mg/ml. Controls were TTR without inhibitor or inhibitor with no TTR. Each tube is incubated at 37°C for 72h. The extent of fibril formation is measured by optical density at 380nm and by a quantitative Congo red binding assay. Colon et al., cited above. Preincubation of the compound (e.g. Flu) with TTR before shifting the pH to amyloid forming conditions exhibits strictly analgous results when the rate for Flu binding to TTR is greater than the denaturation rate to the amyloidogenic intermediate.

Active compounds may be further evaluated by analytical ultracentrifugation to demonstrate that the normal tetrameric structure is adopted in the presence of the inhibitors under acidic conditions that would result in tetramer dissociation and the conformational changes leading to amlyloid formation in the absence of

inhibitor. Miroy et al. *Proc. Nat'l Acad. Sci. USA* 93:15051-15056 (1996); Kelly, *Strucutre* 5:595-600 (1997).

The non-steroidal anti-inflammatory drugs, fenoprofen and flufenamic acid bind to transthyretin, stabilize the non-amyloidogenic tetramer at pH's>4 and inhibit amyloid formation *in vitro*. More specifically, incubation of physiological concentrations of transthyretin at pH 4.4, 37°C (the pH of maximal wild type amyloid fibril formation) resultsn in the conversion of the majority of TTR into amyloid after 72h in the absence, but not in the presence, of 3 eq of flufenamic acid (10.8μM). In the presence of the drug the untracentrifugation data fits nicely to a single species model corresponding to the non-amyloidogenic tetrameric TTR (54kDa). The observed dissociation constant for the $Flu_2 \bullet (TTR)_4$ complex for wild type, V30M and L55P TTR is 148±66nM, 596±383nM and 956±109nM, respectively, as measured by isothermal titration calorimetry, revealing that k_D is lwo enough to saturate TTR when the Flu concentration = 10.8μM, i.e., 3x the phsiological tetramer concentration (3.6μM).

Example 13

TTR-Flu crystal structure

Regarding the crystal structure, co-crystals of flufenamic acid bound to TTR were produced. First crystals of TTR were grown using the hanging drop vapor diffusion method from solutions of 5mg/ml TTR (in 100mM KCl, 100mM phosphate, pH7.4, 1 M ammonium sulfate) equilabrated against 2 M ammonium sulfate. In order to prepare the TTR-drug complex, crystals were soaked for five weeks with a 10-fold molar excess of flufenamic acid to ensure full saturation of both binding sites. The crystal structure of the binary complex was solved using

molecular replacement methods and refined to a resolution of 2.0Å. As in the TTR-T4 complex, two molecules of flufenamic acid were found to bind deeply into the central hormone binding funnels of the TTR tetramer. Flufenamic acid binds into propeller-like conformations which differ in their dihedral angles along the Ar-N-Ar bonds of the drug. The rotation angles ϕ and ϕ' are defined via the atoms C1-C6-N-C1'-C2'; conformation 1: ϕ/ϕ'=-176/39; conformation 2: ϕ/ϕ'=-169/-147.

Due to the two-fold axis along the binding channel of TTR, a statistical disorder model had to be applied. In each binding site four molecules of flufenamic acid with an occupancy of 0.25 have been positioned into their observed density.

The crystallographic data explain both the affinity of the drug towards TTR (able to bind in its low energy solution conformations) as well as the stabilization of the TTR tetramer upon drug binding. In all four binding modes the drug molecule interacts with residues of two adjacent TTR molecules thus mediating several inter-subunit interactions. The CF_3 substituent occupies the innermost halogen binding pocket, interacting with Ser-117, Thr-119, Leu-110 and Ala-108 providing hydrophilic and hydrophobic fluorine contacts (for interaction distances see table II), Figure 1. The biarylamine system of flufenamic acid is placed in a propeller-like conformation into a hydrophobic patch of the T4 binding site between the residues Leu-17 and Ala-108. Finally the carboxylate group on the outer phenyl ring is placed at the entrance of the funnel-shaped binding pocket forming hydrogen bonds with the side chains of two Lys-15 residues.

Binding of flufenamic acid induces a conformational change in TTR, specifically the side

chains of Ser-117 and Thr-119 rearrange to facilitate additional hydrogen bonding between the four subunits, thereby stabilizing the TTR tetramer in addition to what would be expected from simple ligand binding, Figure 2. In the ligand-free tetramer the side chains of all four Ser-117 residues, which are positioned close to the interaction point of the three two-fold axes of the tetramer, are pointing towards the two T4 binding cavities interacting with bulk solvent. Binding of flufenamic acid, however, triggers the side chains of the Ser-117 residues in all four monomers to rotate about 120°, allowing the formation of two non-solvated hydrogen bonds between the Ser-117 residues on adjacent subunits. Furthermore, the induced conformational change of the Thr-119 side chain allows for the formation of a hydrogen bond to an ordered water molecule, which is linked to the carbonyl oxygen of Asp-18 on an adjacent monomer. In summary, Flu binding appears to initiate inter-subunit hydrogen bonding and hydrophobic interactions, the latter being mediated by the arylamine substructure of Flu, which stabilize the quaternary structure of TTR towards pH mediated dissociation and conformation changes associated with amyloidogenesis.

Thus, Flu binds with modest affinity to wild type, L55P and V30M TTR, stabilizing the normally folded state against the pH induced conformational changes that lead to amyloid fibril formation. Since TTR transports only 10-15 % of T4 in plasma (thyroid binding globulin being the main T4 carrier) inhibitor binding to TTR should not perturb thyroid metabolism, especially considering the small effect the TTR knockout has on thyroid metabolism (Palha et al., J. Biol. Chem. 269:33135-33139 (1994).

Example 14

Table II Structure determination.

Intensity data for the apo- and complexed crystal forms of TTR were collected on a DIP2030 imaging plate system (MAC SCIENCE) using a RIGAKU rotating-anode source at 50 kV and 80 mA. The crystals were isomorphous with unit cell dimensions of a = 43.2 Å, b = 85.3 Å, and c = 64.5 Å. They belong to the space group $P2_12_12$, and contain one half of the homo-tetramer in the asymmetric unit. Data were reduced with DENZO and SCALEPACK (Otwinowski, OSCILLATION DATA REDUCTION PROGRAM, PROCEEDINGS OF THE CCP4 STUDY WEEKEND: DATA COLECTION AND PROCESSING (SEERC DARSBURY LABORATORY), 1993) on a Silicon Graphics Indy computer. The protein coordinates from the TTR-bromoflavone complex (Protein Data Bank accession number 1THC) were used as a starting model for the initial refinement of the native structure by molecular dynamics and energy minimization using the program XPLOR (Brunger et al. XPLOR Version 3.1 (Yale U. Press. New Haven CN). Simulated annealing, addition of 56 water molecules and individual temperature factor refinement provided a model which was used to phase a ëcomplex-nativeí difference fourier map. In the resulting map the drug could be located with peaks heights of 10 $_{rms}$ in both binding pockets of the TTR tetramer. A statistical disorder model was applied to account for the twofold crystallographic symmetry of the binding channel. Four molecules of flufenamic acid with half occupancy were positioned into their observed electron density. Four different lowest energy conformations of the drug (with different values for the two C-N-C-C dihedrals) were calculated by the program INSIGHT (BIOSYM INSIGHT II, Molecular Simulations, Inc.) and used as an initial model for the drug in the crystallographic refinement. After a first cycle of simulated annealing using non-strict restraints, 56 water molecules were placed into difference fourier maps.

After another round of slow cooling and subsequent positional and temperature factor refinement the resulting drug conformation was compared against the initial $F_{o,complex}$- $F_{o,apo}$ maps and unbiased annealed omit maps, phased in absence of the drug. Two conformations of the drug were found to fit the density maps and both forms were included into the refinement applying an alternate conformation model. Because of the lack of interpretable electron densities in the final map, the 9N-terminal and 2 C-terminal residues were not included into the final model. Coordinates have been deposited in the Protein Data Bank, Brookhaven.

In Table III, interaction distances in the complex of TTR/Flu are shown. The values for both binding modes of hte drug have been averaged over the two symmetry related monomers and are listed in Å. Both molecular conformations differe by a rotation along the N-C bonds (conformation 1:ϕ/ϕ' = -169/-147. Residues marked with an asterix are from the symmetry related subunit.

TABLE II

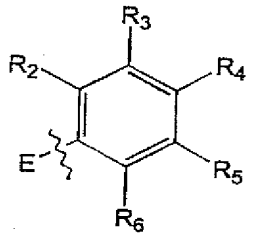
	Apo	TTR*Flu
Resolution (≈)	20-2	20-2
Reflections measured/unique	87447/16489	86716/16664
Completeness (%) overall/outer shell	97.6/79.3	99.6/99.4
R_{sym} (%)* overall/outer shell	6.7/28.9	6.3/28.5
I/σ_I	9.9	10.6
Refinement statistics		
Resolution (≈)**	8-2.2	8-2.0
R_{factor}/R_{free} (%)#∂	18.7/24.5	18.5/24.5
rmsd		
Bond length (≈)	0.014	0.015
Bond angles (degrees)	1.9	2.0

TABLE III

Ligand atom	Protein atom	Distance (Å)	
		Binding mode 1	Binding mode 2
F1	Ser-117 Cβ	3.42	
	Leu-110 N	2.93	
	Leu-110 Cβ	3.07	
	Ser-117 O*		2.66
	Leu-110 N*		2.93
	Leu-110 Cβ*		3.12
F2	Ser-117 Cβ	3.34	
	Leu-110 Cδ2*	3.44	
	Ser-117 Cβ*		3.20
	Leu-110 Cδ2		3.47
F3	Thr-119 Cγ2	3.26	
	Thr-119 N	3.09	
	Ala-108 O	3.01	
	Thr-119 Cγ2*		3.16
	Ala-108 Cβ*		3.75
	Ala-108 O*		3.15
C5'	Leu-110 Cβ	3.93	
C3	Val-121 Cγ2		3.89
	Thr-106 Cγ2	3.92	3.86
C4	Ala-108 Cβ	3.28	3.33
	Val-121 Cγ2	3.89	3.76
	Thr-119 Cγ2	3.68	3.71
	Leu-17 Cδ1*	3.37	3.35
C5	Ala-108 Cβ	3.39	3.20
	Val-17 Cδ1*	2.89	2.98
	Thr-119 Cγ2	3.56	3.53
O1	Lys-15 N	2.98	2.95
	Lys-15 N *	3.59	3.33

CLAIMS

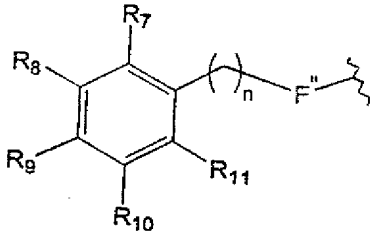
1. A method for treating a human amyloid disease, comprising administering a pharmaceutically effective amount of a composition including an amyloidogenic protein- stabilizing compound; said amyloidogenic protein-stabilizing compound has one of the formulas (1a), (1a'), (1b), or (1c) below:



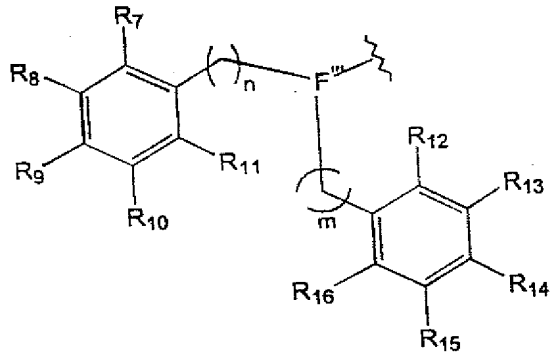
(1a)

wherein E has formula (i), (ii) or (iii):

(i) $-F'-R_{17}$;



(ii)



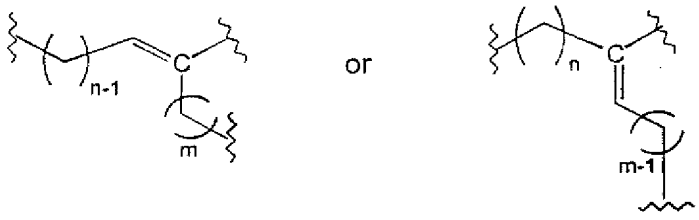
(iii)

in which

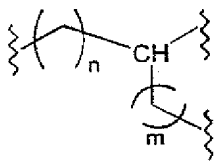
F' is NR_a, O, or S;

F" is NR_a, O, S, CH_2, $Si(R_b)(R_c)$, $-PR_b$;

F''' is N, sp^2 hybridized C as shown in formula (q1) or (q2) or sp^3 hybridized C as shown in formula (q3)



(q1) (q2) ;



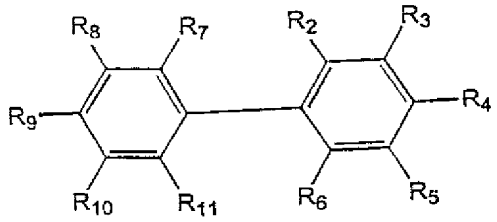
(q3)

and in which:

- R_a is H or C_{1-6} alkyl;
- each of R_b and R_c is independently C_{1-6} alkyl, provided that at least two of R_1 - R_{17} are each independently selected from halo, C_{0-8} hydroxyalkyl, or -A-B, wherein A is branched or straight C_{0-6} alkylene, C_{1-6} acyl, or C_{1-6} aminoalkylene; B is R_d, $-COOR_d$, $-OR_d$, $-SR_d$, $-NHR_d$, or $-N(C_{1-3}$ alkyl$)(R_d)$, R_d being -H, straight or branched C_{1-8} alkyl, C_{2-8} heterocyclic radical, C_{2-8} heteroaryl, or (C_{0-4} alkyl)phenyl; and the remaining R_1 - R_{17} are independently selected from -H, C_{1-6} alkyl, C_{1-6} haloalkyl, benzyl, C_{1-8} alkene, C_{3-8} cycloalkyl, $-N^+R_fR_gR_h$,
- each of R_f, R_g, and R_h being independently selected from -H and C_{1-10} alkyl, (C_{0-4} alkyl)-SO_3H, -(C_{0-4} alkyl)-PO_3H_2, -(C=O)(C_{1-5} alkyl), and $-R_j-M-R_k$, where R_j is straight or branched C_{1-10} alkylene; R_k is H, or straight or branched C_{1-10} alkyl; and M is carbonyl or a heteroatom; or R_j-M-R_k taken together are C_{4-10} cycloalkyl, C_{2-8} heterocyclic radical, or C_{2-8}

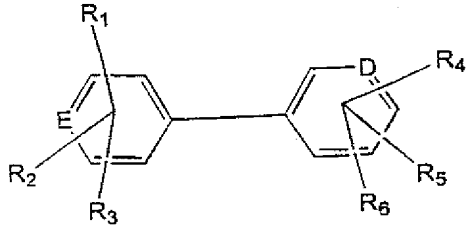
heteroaryl; each of n and m is independently an integer between 0 and 2, inclusive;

(1a'),



in which R_2 - R_{11} are the same as defined above for formual (1a);

(1b)

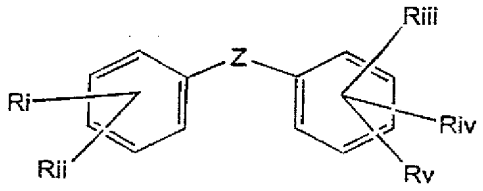


wherein

- each of D and E is independently selected from C, N, O, and S;
- no more than two of R_1 - R_6 are independently selected from -A-B, where A is branched or straight C_{0-6} alkylene, C_{1-6} acyl, or C_{1-6}

aminoalkylene; and B is R_a, $-COOR_a$, $-OR_a$, $-SR_a$, NHR_a, or $N(C_{1-3}$ alkyl$)(R_a)$, R_a being H, straight or branched C_{1-8} alkyl, C_{2-8} heterocyclic radical, C_{2-8} heteroaryl, or $(C_{0-4}$ alkyl$)$phenyl;

- the remaining R_1 - R_6 are independently selected from H, halo, hydroxyl, and cyano;



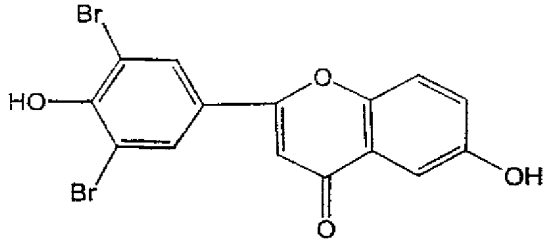
(1c)

wherein

- Z is O or N(G);
- G is selected from H, C_{1-8} alkyl, C_{5-10} cycloalkyl, C_{6-10} aryl, C_{2-8} heterocyclic radical; G being optionally substituted with one or more halo, hydroxy, carboxyl, C_{1-2} haloalkyl, or cyano groups;
- each of R_i - R_v is independently selected from hydrogen, halo, hydroxy, C_{1-5} alkyl, C_{1-5} haloalkyl, cyano, and -A-B as described in formula (1a) above;

2. A method for treating a human amyloid disease, comprising administering a pharmaceutically

effective amount of a composition including an amyloidogenic protein- stabilizing compound; said amyloidogenic protein-stabilizing compound is a nonsteroidal anti-inflammatory compound selected from 2,4,6-triiodophenol, mefenamic acid, diflunisal, meclofenamic acid, milrinone, ethacrynic acid, diclofenac, indomethacin, sulindac, fenclofenac, fenoprofen, flufenamic acid, and EMD 21388 (Merck), (i.e., 2-(3,5-dibromo-4-hydroxyphenyl)-4H-6-hydroxy-1-benzopyran-4-one, see structure below).



3. A method of claim 1 or claim 2, wherein the disease is selected from primary systemic amyloidosis, senile systemic amyloidosis, familial amyloid polyneuropathy I, hereditary cerebral amyloid angiopathy, hemodialysis-related amyloidosis, familial amyloid polyneuropathy III, Finnish hereditary systemic amyloidosis, Type II diabetes, medullary carcinoma of the thyroid, spongiform encephalopathy, atrial amyloidosis, hereditary non-neuropathic systemic amyloidosis, injection-localized amyloidosis, and hereditary renal amyloidosis.

4. A method of claim 3, wherein the disease is senile systemic amyloidosis or familial amyloid polyneuropathy I.

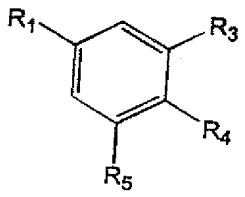
5. A method of claim 1 or claim 2, wherein the disease is Alzheimer's disease.

6. A method of claim 1 or claim 2, wherein the disease is a spongiform encephalopathy.

7. A method of claim 1 or claim 2, wherein the amyloidogenic protein is transthyretin or a variant thereof.

8. A method of claim 1 or claim 2, wherein the compound has the formula (1a).

9. A method of claim 1 or claim 2, wherein the compound has the formula (1d):



(1d)

wherein

- at least one of R_1 and R_4 is selected from F, Cl, Br, I, hydroxy, C_{1-6} haloalkyl (e.g., trifluoromethyl), C_{1-8} alkyl, and -A-B, A being branched or straight C_{0-6} alkylene, C_{1-6} acyl, or C_{1-6} aminoalkylene, and B being R_a, -$COOR_a$, -OR_a, -SR_a, NHR_a, or $N(C_{1-3}$ alkyl$)(R_a)$, R_a being H, straight or branched C_{1-8} alkyl,

C_{2-8} heterocyclic radical, C_{2-8} heteroaryl, or (C_{0-4} alkyl)phenyl;

and

- each of R_3, R_5, R_6, and any remaining R_1 or R_4 is independently selected from H, halo (e.g., I or Br), hydroxyl, C_{2-8} heterocyclic radical, C_{2-8} heteroaryl, and cyano.

10. A method of claim 1, wherein the compound has the formula (1b).

11. A method of claim 1, wherein the compound has the formula (1c).

12. A method of claim 8 wherein the compound is fenoprofen.

13. A method of claim 8, wherein the compound is flufenamic acid.

14. A method of claim 1 or claim 2, wherein the compound binds to transthyretin *in vitro* with a higher affinity than it binds to a human serum binding protein.

15. A method of claim 14, wherein the serum binding protein is thyroxine binding globulin.

16. A method of claim 14, wherein the serum binding protein is serum albumin.

17. A method of claim 1 or claim 2, wherein the compound binds to transthyretin *in vitro* with a higher affinity than it binds to thyroid hormone receptor.

18. An amyloidogenic protein-drug conjugate, comprising an amyloidogenic protein reversibly bound to a compound of formulae (1a), (1a'), (1b), (1c), or (1d).

19. A conjugate of claim 18, wherein the compound is a compound of formula (1c).

20. A conjugate of claim 18, wherein the compound is a biphenylether, a biphenylthioether or a biphenylamine.

21. A conjugate of claim 18, wherein the compound is fenoprofen or flufenamic acid.

22. A method for inhibiting amyloid fibril formation, comprising

forming an amyloidogenic protein/drug conjugate comprising

(a) a compound selected from formula (1a), (1a'), (1b), (1c), and the nonsteroidal anti-inflammatory compounds 2,4,6-triiodophenol, mefenamic acid, diflunisal, meclofenamic acid, milrinone, ethacrynic acid, diclofenac, indomethacin, sulindac, fenclofenac, fenoprofen, flufenamic acid, and EMD 21388 (Merck), (i.e., 2-(3,5-dibromo-4-hydroxyphenyl)-4H-6-hydroxy-1-benzopyran-4-one), or a metabolite thereof (e.g., esters, amines, ammonium salts and Tris salts), and

(b) an amyloidogenic protein reversibly bound to the compound.

23. The method of claim 22, wherein the drug of the amyloidogenic protein/drug conjugate is flufenamic acid.

24. A method for stabilizing an amyloidogenic protein, comprising administering a pharmaceutically effective amount of a composition including an amyloidogenic protein-stabilizing compound having a formula selected from formula (1a), (1a'), (1b), (1c), and the nonsteroidal anti-inflammatory compounds 2,4,6-triiodophenol, mefenamic acid, diflunisal, meclofenamic acid, milrinone, ethacrynic acid, diclofenac, indomethacin, sulindac, fenclofenac, fenoprofen, flufenamic acid and EMD 21388 (Merck), (i.e., 2-(3,5-dibromo-4-hydroxyphenyl)-4H-6-hydroxy-1-benzopyran-4-one), or a metabolite thereof (e.g., esters, amines, ammonium salts and Tris salts).

25. A method of claim 24, wherein the amyloidogenic protein-stabilizing compound is flufenamic acid.

26. A method of claim 24, further comprising the step of forming an amyloidogenic protein/drug conjugate comprising

(a) a compound selected from formula (1a), (1a'), (1b), (1c), and the nonsteroidal anti-inflammatory compounds 2,4,6-triiodophenol, mefenamic acid, diflunisal, meclofenamic acid, milrinone, ethacrynic acid, diclofenac, indomethacin, sulindac, fenclofenac, flufenamic acid, and EMD 21388 (Merck), (i.e., 2-(3,5-dibromo-4-hydroxyphenyl)-4H-6-hydroxy-1-benzopyran-4-one), or a metabolite thereof (e.g., esters, amines, ammonium salts and Tris salts), and

(b) an amyloidogenic protein reversibly bound to said compound.

27. The method of claim 26, wherein the drug of the amyloidogenic protein/drug conjugate is flufenamic acid.

28. A medicament for treating a human amyloid disease comprising the compound of claim 1 or claim 2 in a pharmaceutically acceptable carrier.

29. A method of making a medicament for treating a human amyloid disease comprising mixing the composition of claim 1 or claim 2 with a pharmaceutically acceptable carrier.

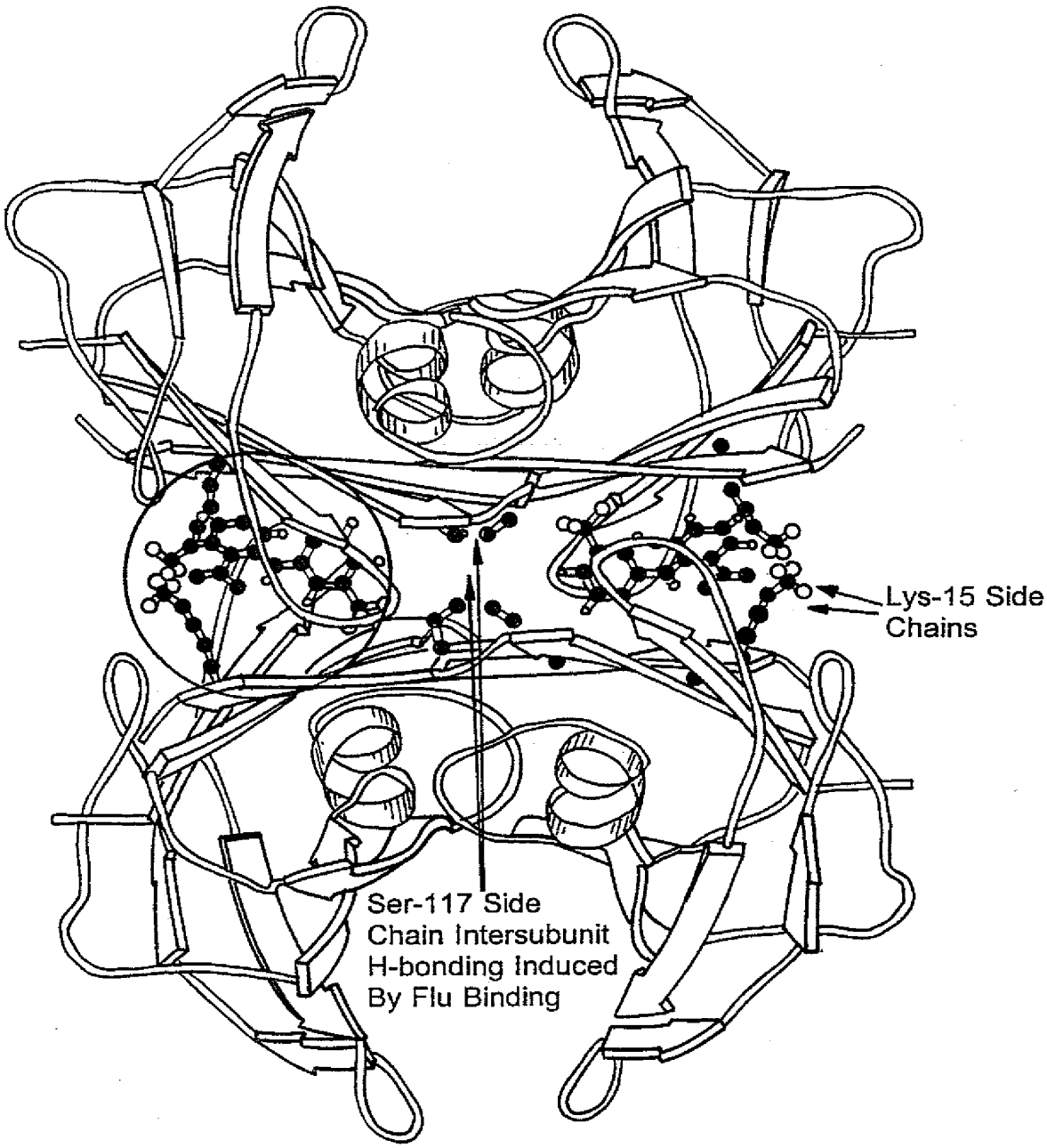


FIG. 2

CORRECTED VERSION*

CORRECTED VERSION**

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(71) Applicant *(for all designated States except US)*: TEXAS A & M UNIVERSITY [US/US]; 3110 Wisenbaker, College Station, TX 77843 (US).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: DE GUZMAN MIROV, Greta, J. [PH/PH]; 2810 E Zobel Street, Makati City 1200 (PH). KELLY, Jeffery, W. [US/US]; 213 Chimney Hill, College Station, TX 77840 (US). LAI, Zhihong [CN/US]; 3216 Hunter's Glen Drive, Plainsboro, NJ 08536 (US). LASHUEL, Hilal, A. [US/US]; Apartment 6, 198 Court Street, Brooklyn, NY 11201 (US). PETERSON, Scott, A. [US/US]; 3723 Cobblestone Place, Topeka, KS 66610 (US).

(74) Agent: FREEMAN, John, W.; Fish & Richardson P.C., 225 Franklin Street, Boston, MA 02110–2804 (US).

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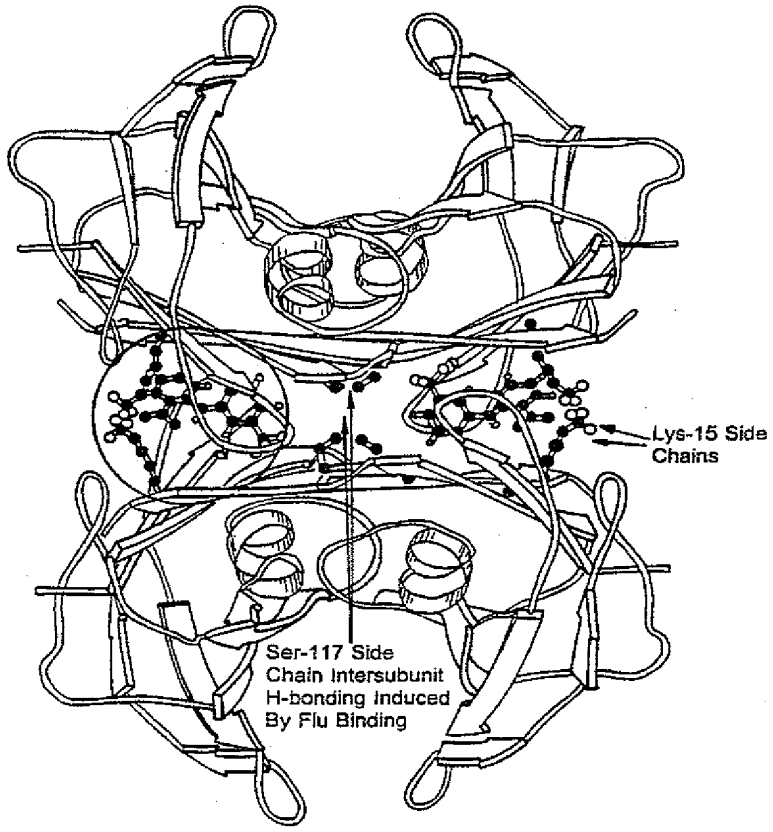
(57) Abstract

A method for treating a human amyloid disease which includes administering a pharmaceutically effective amount of a composition including an amyloidogenic protein-stabilizing aryl compound. These compounds are selected from 2,4,6-triiodophenol, mefenamic acid, diflunisal, meclofenamic acid, milrinone, ethacrynic acid, diclofenac, indomethacin, sulindac, fenclofenac, fenoprofen, flufenamic acid, and EMD 21388 (Merck), (i.e., 2-(3,5-dibromo-4-hydroxyphenyl)-4H-6-hydroxy-1-benzopyran-4-one).



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INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 97/24181

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 A61K31/405 A61K31/055

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	ROGERS ET AL.: "Clinical trial of indomethacin in Alzheimer's disease" NEUROLOGY, vol. 43, 1993, pages 1609-1611, XP002070235 see abstract see page 1609, column 2, paragraph 3 - page 1610, column 2, paragraph 1; table 1 ---	1,2
X	GOTTSCHALK P.E.: "beta-amyloid induction of gelatinase B secretion in cultured microglia: inhibition by dexamethasone and indomethacin" NEUROREPORT, vol. 7, no. 18, 1996, pages 3077-3080, XP002070236 see abstract, see conclusions ---	1,2

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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2 July 1998	25. 11. 98

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European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	A. Jakobs

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INTERNATIONAL SEARCH REPORT

Inter ional Application No
PC I/US 97/24181

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	VAN MUISWINKEL ET AL.: "Do Nosteroidal Anti-Inflammatory Drugs Have a Protective Effect Against Dementia" DRUGS & AGING, vol. 9, no. 1, 1996, pages 1-7, XP002070237 see page 3, column 1, paragraph 3 - page 4, column 1, paragraph 3 ---	1,2
X	ZHANG ET AL.: "INHIBITION OF TRANSGLUTAMINASE-INDUCED POLYMERIZATION OF AMYLOID beta-PEPTIDE BY PHARMACOLOGIC AGENTS" PHARMACEUTICAL RESEARCH, vol. 13, no. 9 Suppl., 1996, page S123 XP002070238 see the whole document ---	1,2
X	MCGEER ET AL.: "Anti-inflammatory drugs and Alzheimer disease" THE LANCET, vol. 335, no. 8696, 1990, page 1037 XP002070239 see column 2, paragraph 2-3 ---	1,2
X	US 5 192 753 A (MCGEER PATRICK L ET AL) 9 March 1993 see abstract see column 5, line 16 - column 66, line 9; claims 1-6 ---	1
X	BREITNER J.C.S.: "The role of Anti-inflammatory Drgs in the revention and treatment of Alzheimer's disease" ANN. REV. MEDICINE, vol. 47, 1996, pages 401-411, XP002070240 see page 403, paragraph 1 - page 405, paragraph 1 ---	1,2
X	BREITNER ET AL.: "Inverse association of anti-Inflammatory treatment and Alzheimer's disease" NEUROLOGY, vol. 44, 1994, pages 227-232, XP002070241 see abstract; tables 1-3 ---	1,2
X	AISEN ET AL.: "Inflammatory Mechanism in Alzheimer's Disease: Implications for Therapy" AM. J. PSYCHIATRY, vol. 151, no. 8, 1994, pages 1105-1113, XP002070242 see abstract see page 1109, column 1, paragraph 3 - page 1110, column 2, paragraph 1 --- -/--	1,2

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INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 97/24181

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	RICH ET AL.: "Nonsteroidal anti-Inflammatory drugs in Alzheimer's disease" NEUROLOGY, vol. 45, 1995, pages 51-55, XP002070243 see abstract see page 52, column 1, paragraph 4 - page 54, column 1, paragraph 4; tables 1,2 ---	1,2
X	FAGARASAN ET AL.: "IL-1 and anti-inflammatory drugs modulate A beta cytotoxicity in PC12 cells" BRAIN RESEARCH, vol. 723, no. 1,2, 1996, pages 231-234, XP002070244 see abstract; tables 1,2 ---	1,2
X	TATE ET AL.: "INDOMETHACIN REVERSES beta-AMYLOID INDUCED GLIOSIS" SOC. NEUROSC. ABSTR., vol. 21, no. 1-3, 1995, page 1480 XP002070245 see the whole document ---	1,2
X	MCKINNEY ET AL.: "Molecular Interaction of Toxic Chlorinated Dibenzo-p-dioxins and Dibenzofurans with Thyroxine Binding Prealbumin" J. MED. CHEM., vol. 28, no. 3, 1985, pages 375-381, XP002070246 see abstract see page 377, column 1, paragraph 3 - page 378, column 1, paragraph 1 ---	18,22, 24,26
X	WO 95 22992 A (CHIRON CORP) 31 August 1995 see abstract see page 7, paragraph 4; claims 1-17; example 2 ---	1,2,7-9, 14-18, 24,26,29
X,P	MIROY ET AL.: "Inhibiting transthyretin amyloid fibril formation via protein stabilization" PROC. NATL. ACAD. SCI. USA, vol. 93, no. 26, 1996, pages 15051-15056, XP002070247 see abstract see page 15053, column 1, paragraph 2 - page 15056, column 1, paragraph 1; figures 1-5 --- -/--	1-5,7-9, 14-18, 24,26

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INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 97/24181

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP 0 675 103 A (SCOTIA HOLDINGS PLC) 4 October 1995 see abstract; claims 1-8	1,2

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Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

mational application No.
PCT/US 97/ 24181

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
See INFORMATION SHEET PCT/ISA/210

2. [X] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
see INFORMATION SHEET PCT/ISA/210

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [X] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

1-8, 14-18, 22,24,26,28,29 (all partially)

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Allthough claims 1-17, 22-27, 29 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

Further defects under Article 17(2)(a)

In view of the large number of compounds, which are defined by the general definition in the independent claims, the search had to be restricted for economic reasons. The search was limited to the compounds for which pharmacological data was given and/or the compounds mentioned in the claims, and to the general idea underlying the application. (see Guidelines, Chapter III, paragraph 2.3).
Claim 12 is not clear because fenoprofen does not belong to formula Ia because not at least two substitutents are different from hydrogen

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

1. Claims: 1-8, 14-18,22,24,26,28,29 (all partially)

Use of the compounds of formula 1a(i) (e.g. triiodophenol) for the treatment of human amyloid disease.

2. Claims: 1-8, 14-18,22,24,26,28,29 (all partially), 21,23, 25,27

Use of the compounds of formula 1a(ii) (e.g. diclofenac, mefenamic acid, meclofenamic acid, flufenamic acid) for the treatment of human amyloid disease.

3. Claims: 1-8, 14-18,22,24,26,28,29 (all partially), 13

Use of the compounds of formula 1a(iii) for the treatment of human amyloid disease.

4. Claims: 1-7, 14-18,22,24,26,28,29 (all partially)

Use of the compounds of formula 1a' (e.g. diflunisal) for the treatment of human amyloid disease.

5. Claims: 1-7, 14-18,22,24,26,28,29 (all partially), 10

Use of the compounds of formula 1b (e.g. milrinone) for the treatment of human amyloid disease.

6. Claims: 1-7, 14-18,22,24,26,28,29 (all partially), 11,12, 19-21

Use of the compounds of formula 1c (e.g. fenoprofen, fenclofenac) for the treatment of human amyloid disease.

7. Claims: 1-7, 14-18,22,24,26,28,29 (all partially), 9

Use of the compounds of formula 1d (e.g. EMD-21388) for the treatment of human amyloid disease.

8. Claims: 2-7,14-17,22,24,26,28,29 (all partially)

Use of sulindac for the treatment of human amyloid disease.

9. Claims: 2-7,14-17,22,24,26,28,29 (all partially)

Use of indomethacin for the treatment of human amyloid

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

disease.

10. Claims: 2-7,14-17,22,24,26,28,29 (all partially)

Use of ethacrynic acid for the treatment of human amyloid disease.

INTERNATIONAL SEARCH REPORT

formation on patent family members

Inter··tional Application No
PC·/US 97/24181

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 5192753 A	09-03-1993	WO 9324115 A	09-12-1993
		EP 0642336 A	15-03-1995
WO 9522992 A	31-08-1995	AU 1843295 A	11-09-1995
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(71) Applicant *(for all designated States except US)*: **TEXAS A & M UNIVERSITY** [US/US]; 310 Wisenbaker, College Station, TX 77843 (US).

(72) Inventors; and

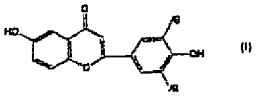
(75) Inventors/Applicants *(for US only)*: **DE GUZMAN MIROV, Greta, J.** [PH/PH]; 2810 E Zobel Street, Makati City 1200 (PH). **KELLY, Jeffery, W.** [US/US]; 213 Chimney Hill, College Station, TX 77840 (US). **LAI, Zhihong** [CN/US]; 3216 Hunter's Glen Drive, Plainsboro, NJ 08536 (US). **LASHUEL, Hilal, A.** [US/US]; Apartment 6, 198 Court Street, Brooklyn, NY 11201 (US). **PETERSON, Scott, A.** [US/US]; 3723 Cobblestone Place, Topeka, KS 66610 (US).

(74) Agent: **FREEMAN, John, W.**; Fish & Richardson P.C., 225 Franklin Street, Boston, MA 02110-2804 (US).

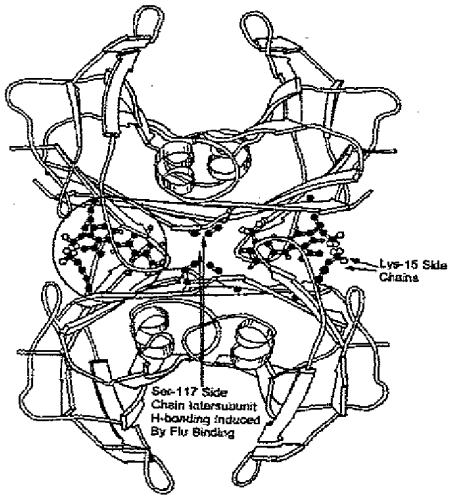
(81) Designated States *(national)*: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES,

[Continued on next page]

(54) Title: ANTI-AMYLOIDOGENIC AGENTS



(I)



(57) Abstract: A method for treating a human amyloid disease which includes administering a pharmaceutically effective amount of a composition to form an amyloidogenic protein-stabilizing aryl compound. These compounds include an amyloidogenic protein-stabilizing compound; said amyloidogenic protein-stabilizing compound is a nonsteroidal anti-inflammatory compound selected from 2,4,6-triiodophenol, mefenamic acid, diflunisal, meclofenamic acid, milrinone, ethacrynic acid, diclofenac, indomethacin, sulindac, fenclofenac, fenoprofen, flufenamic acid, and EMD 21388 (Merck), (i.e., 2-(3,5-dibromo-4-hydroxyphenyl)-4H-6-hydroxy-1-benzopyran-4-one see structure (I)).

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FI, GB, GE, GH, GM, GW, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW.

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